

## REMARKS

Upon entry of the amendment, claims 1-27 and 32 will be pending in the application. Claims 28-31 and 33-38 are cancelled without prejudice as drawn to non-elected subject matter. Applicants reserve the right to pursue the subject matter of all cancelled claims in a continuing application.

Applicants have amended the specification as requested by the Examiner. The amendment replacing the paragraphs beginning at page 35, line 31 to page 39, line 5 with the indicated text removes inadvertent page breaks present in the application as filed. The specification has also been amended to delete reference to an unidentified sequence number in the paragraph beginning at page 8, line 7.

In response to the Notice to Comply with Sequence Listing Requirements, enclosed with the March 25, 2004 Office Action, Applicants submit a copy of the Notice to Comply, a substitute a paper copy of the Sequence Listing (pages 1-16), a substitute computer readable form (CRF) of the "Sequence Listing" (1 disk), and a Statement in Support of Computer Readable Form Submission. The application has been amended to replace the pending sequence listing.

Applicants enclose a new Combined Declaration/Power of Attorney for Matthew Bahamonde (5 pages) in compliance with 37 CFR 1.67(a).

No new matter has been added by these above amendments.

### Rejections under 35 USC 112, first paragraph

Claims 1-27 and 32 are rejected for lack of enablement. The rejection is traversed.

In the paragraph bridging pages 5 and 6, the Examiner states

Applicants have provided no *in vitro* or *in vivo* experimental systems to demonstrate that application of any specific inhibitor of BMP-3 expression or activity will result said results [reducing the severity of bone fractures, reducing

the incidence of a bone fracture and a method for treating osteoporosis in which a subject is provided an agent that inhibits BMP-3 expression or activity].

Applicants respectfully disagree that *in vitro* or *in vivo* data are necessary to enable the claimed methods. As is explained in detail below, the teachings of the specification, coupled with the knowledge available to one of ordinary skill in the art, allow the artisan to practice the full scope of the claimed invention without undue experimentation. To the extent the Examiner requires *in vivo* data to prove that applicants have taught how to use the claimed invention, such a demand is improper. In the context of the utility requirement, *in vivo* data are not per se required to establish a therapeutic utility for a claimed invention. The MPEP states at section 2107.03 (8<sup>th</sup> Ed., first revision):

The Federal courts have consistently reversed rejections by the Office asserting a lack of utility for inventions claiming a pharmacological or therapeutic utility where an applicant has provided evidence that reasonably supports such a utility. In view of this, Office personnel should be particularly careful in their review of evidence provided in support of an asserted therapeutic or pharmacological utility.

In the present application, the claimed methods are based on the discovery that a reduction in BMP-3 expression by genetic ablation of the gene encoding BMP-3 leads to increased bone density (see, e.g. page 2, lines 6-12 and Example 6 at pages 43-44 of the specification). In the present case, standard clinical tests were used to evaluate BMP-3-deficient mice. Importantly, a novel skeletal phenotype was seen in adult *Bmp3* mutants. Radiographic analyses revealed an increased bone density in femurs from 5-6 week-old *Bmp3*<sup>-/-</sup> mice compared to WT littermates. Histomorphometric analyses confirmed that mutants exhibit increased trabecular metaphyseal bone density. Total trabecular bone volume in mutants is twice that of WT littermates. The biochemical data obtained using such tests showed that a decrease in BMP-3 correlated with increased bone density.

Applicants also provide in the specification additional evidence supporting a role for BMP-3 in antagonizing bone growth, and that BMP-3 effects this by interacting with BMP-2. For example, applicants show in Example 2 on page 40, line 19 to page 41, line 2 that injection of BMP-3 mRNA into *Xenopus* embryos produces dorsalization, which is consistent with a role for BMP-3 as an antagonist of endogenous BMPs. A specific interaction between BMP-3 and BMP-2 function *in vitro* is provided in Example 3, at page 41, lines 4-14, which shows BMP-3 antagonizes BMP-2 function *in vitro* in osteoprogenitor cells. BMP-2 induces alkaline phosphatase (ALP) and osteocalcin (OC) induction in the absence, but not in the presence of BMP-3. Applicants demonstrate further in Example 4 (page 41, line 16 to page 42, line 13) that BMP-3 can abolish BMP-2 induced Msx2 reporter expression in C3H 10T1/2 cells. Also shown are the results of studies in Example 5 (page 42, lines 15-29) that BMP-3 induces the expression of TGF $\beta$ /activin-responsive genes but not BMP-responsive genes.

Knockout mice such as the BMP-3 knockout mice described in the specification are valuable tools for discovering the function(s) of genes, and for identifying methods of treatments based on the functions of the genes. This premise is well known and accepted in the art. For example, the RANKL gene has been identified as a critical factor in the differentiation and activation of osteoclasts, which are cells that mediate bone resorption (reviewed in Katagiri et al., Oral Diseases 8:147-59, 2002; included as Exhibit A). RANKL acts by binding to RANK, a cell surface receptor molecule that is present on pre-osteoclasts and osteoclasts. Mice with a disrupted RANKL gene lack osteoclasts and show severe osteopetrosis, or abnormally high bone density. The phenotype of mice with disrupted RANKL genes suggests that conditions associated with unwanted bone loss can be treated by administering to an agent that inhibits RANKL expression or activity. Accordingly, Bekker et al., J. Bone and Mineral Res. 19:1059-

66, 2004 (included as Exhibit B) have described a monoclonal antibody that prevents binding of RANKL to RANK and which is useful in preventing osteoporosis in post-menopausal women. Data obtained from such knockout models thus establish a “cause-and-effect” relationship between the expression of the gene product and a function of the gene product.

The pending claims are drawn to a method for reducing the severity of a bone fracture in a subject by administering to a site of the bone fracture in the subject a therapeutically effective amount of an agent that inhibits activity or expression of a BMP-3 polypeptide (see claim 1, from which depends claims 2-20). Also pending are claims drawn to a method treating osteoporosis, a method of reducing the risk of fracture using these agents, as well as pharmaceutical compositions including these agents (claims 21, 22, 23 (and its dependent claims 24-27), and a claim to method of antagonizing BMP-2 activity in host, by administering to the subject an agent that increases activity of BMP-3 in the host (claim 32).

As was reviewed by the Examiner in the Office Action, the enablement standard requires that the specification provide a description that, when coupled with the knowledge possessed by a person of ordinary skill in the art, enables that person to make and use the claimed invention.<sup>1</sup> The factors to be analyzed in determining whether undue experimentation is required to practice the full scope of the claims are discussed in In re Wands.<sup>2</sup> The court in In re Wands set forth eight factors to be considered in determining whether undue experimentation would be required: (1) the state of the prior art, (2) the predictability or unpredictability of the art, (3) the breadth of the claims (4) the presence or absence of working examples, (5) the amount of direction or guidance presented, (6) the relative skill of those in the prior art, (7) the nature of the invention,

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<sup>1</sup> Atlas Powder Co. v. E.I. duPont De Nemours & Co., 750 F.2d 1569, 1576 (Fed. Cir. 1984).

<sup>2</sup> In re Wands, 858 F.2d 731, 736-7 (Fed. Cir. 1988).

and (8) the quantity of experimentation necessary. “The key word is ‘undue,’ not ‘experimentation.’”<sup>3</sup>.

Applicants submit that on balance the Wands factors argue in favor of the enablement of the claimed methods. In several respects, the state of the prior art is high. The use of other bone morphogenetic proteins (BMP’s) to modulate bone growth is well known in the art (reviewed in Katagiri et al. (Exhibit A); and Syakaras et al., J. Oral Science 45:57-73, 2003; included as Exhibit C). In addition, as noted above for anti-RANK antibodies, monoclonal antibodies have been used to disrupt *in vivo* specific functions, which are initially inferred by the phenotype resulting from genetic ablation experiments, associated with bone metabolism.

Many biological reagents for use in the claims are either known in the art or can be made using methods well known in the art. Human BMP-3 nucleic acid and polypeptide sequences are known and are disclosed in the specification (see, e.g., page 34, line 16 through page 35, line 15 of the specification). Methods of making antibodies are taught in detail in the specification at, e.g., p. 7, line 24 to page 18, line 24. Soluble activin receptor-based inhibitors are discussed at, e.g., page 18, line 26 to p. 23, line 25, as are nucleic acid-based agents at, e.g., page 23, line 26 to page 28, line 19. Pharmaceutical compositions containing the agents and methods for delivering the agents are taught at, e.g., page 28, line 21 to page 33, line 28.

In addition, the level of skill of one in the art is high. The skilled artisan in the relevant field is a molecular biologist or medical doctor and thus has a high level of skill. As has been discussed above, bone morphogenetic proteins have been used to treat bone disorders for several years. Moreover, BMP-3–based agents required by the claims can be readily made by such an artisan.

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<sup>3</sup> In re Angstadt, 537 F. 2d 498, 504 (C.C.P.A. 1976).

Also weighing in favor of enablement is the nature of the invention. The nature of the invention is not complex. The steps of the claimed method and reagents for performing the methods are well within the skill of the artisan. The specific indications recited in the claims—bone fracture and osteoporosis, are easily ascertained by one of ordinary skill in the art. Moreover, as is noted by the Examiner, the specification provides extensive guidance for making (see, e.g., the specification at page 7, line 24 through page 26, line 14) and using (see, e.g., the specification at page 28, line 22 through page 30, line 8 and page 30, line 9 to page 33, line 16) agents that inhibit BMP-3 activity.

While some experimentation may be required for testing agents that fall within the scope of the claims, this does not equate to a finding that the claims are not enabled. In Wands, the Court stated:

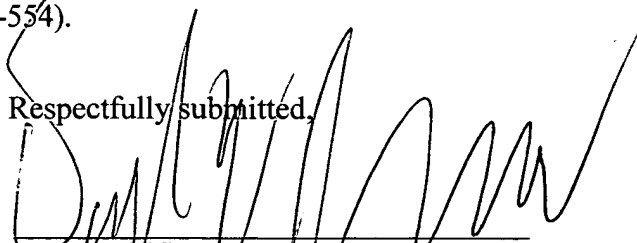
[A] considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

Applying this criterion here, the techniques required to practice the claimed methods were described in the specification or were known to those skilled in the art as of the filing date.

The correlation between bone mass and BMP-3 expression has been established in the mouse genetic ablation studies. Contrary to the Examiner's characterization of the claimed methods, Applicant submits that the methods are not complex. The sequences of the human BMP-3 gene and gene product are known, as are methods for designing agents based on these sequences. Thus, Applicants submit that the undue experimentation would not be required of one skilled in the art to make and use reagents required for treating the indications recited by the claims.

Applicants submit that the application is in condition for allowance, and such action is requested. A petition for extension of time accompanies this response. The Commissioner is authorized to charge any additional fees that may be required, or credit any overpayment of same, to Deposit Account No. 50-0311 (Reference No. 22058-554).

Respectfully submitted,



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## REVIEW ARTICLE

### Bone Biology

# Regulatory mechanisms of osteoblast and osteoclast differentiation

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Bone is continuously destroyed and reformed to maintain constant bone volume and calcium homeostasis in vertebrates throughout their lives. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Recent developments in bone cell biology have greatly changed our conceptions of the regulatory mechanisms of the differentiation of osteoblasts and osteoclasts. Bone morphogenetic proteins (BMPs) play critical roles in osteoblast differentiation. The discovery of Smad-mediated signals revealed the precise functions of BMPs in osteoblast differentiation. Transcription factors, Runx2 and Osterix, are found to be essential molecules for inducing osteoblast differentiation, as indicated by the fact that both Runx2-null mice and Osterix-null mice have neither bone tissue nor osteoblasts. Smad transcriptional factors are shown to interact with other transcription regulators, including Runx2. Also, the recent discovery of receptor activator of NF- $\kappa$ B ligand (RANKL)–RANK interaction confirms the well-known hypothesis that osteoblasts play an essential role in osteoclast differentiation. Osteoblasts express RANKL as a membrane-associated factor. Osteoclast precursors that express RANK, a receptor for RANKL, recognize RANKL through the cell–cell interaction and differentiate into osteoclasts. Recent studies have shown that lipopolysaccharide and inflammatory cytokines such as tumor necrosis factor receptor- $\alpha$  and interleukin 1 directly regulate osteoclast differentiation and function through a mechanism independent of the RANKL–RANK interaction. Transforming growth factor- $\beta$  super family members and interferon- $\gamma$  are also shown to be important regulators in osteoclastogenesis. These findings have opened new areas for exploring the

molecular mechanisms of osteoblast and osteoclast differentiation.

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**Keywords:** osteoblast; osteoclast; bone morphogenetic proteins; Smad; receptor activator of nuclear factor  $\kappa$ B ligand; macrophage colony-stimulating factor

## Introduction

Bone is continuously destroyed and reformed in vertebrates in order to maintain bone volume and calcium homeostasis throughout their lives. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Osteoblasts produce bone matrix proteins including type I collagen, the most abundant extracellular protein of bone, and also take charge of mineralization of the tissue (Aubin and Triffitt, 2002). Osteoblasts, chondrocytes, myocytes and adipocytes are all derived from a common progenitor called undifferentiated mesenchymal cells. During the process of their differentiation, progenitor cells acquire specific phenotypes under the control of respective regulatory factors. Bone morphogenetic proteins (BMPs) play critical roles in the differentiation of undifferentiated mesenchymal cells into osteoblasts. Recent studies have elucidated the molecular mechanism of osteoblast differentiation induced by BMP.

Osteoclasts are multinucleated cells responsible for bone resorption. The most characteristic feature of osteoclasts is the presence of ruffled borders and clear zone (Väänänen and Zhao, 2002). Vacuolar H<sup>+</sup>-ATPase exists in the ruffled border membrane of osteoclasts, and acidifies resorbing area under the ruffled border. The ruffled border is surrounded by a clear zone, which serves for the attachment of osteoclasts to the bone surface to maintain a microenvironment favorable for bone resorption. Osteoclasts are differentiated from hemopoietic cells of the monocyte/macrophage lineage

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under the control of bone microenvironments. Osteoblasts or bone marrow stromal cells have been shown to regulate osteoclast differentiation providing the microenvironment similar to bone. The recent discovery of new members of the TNF receptor-ligand family has clarified the molecular mechanism of osteoclast differentiation regulated by osteoblasts/stromal cells. This review article describes the current knowledge of the mechanisms of the regulation of osteoblast and osteoclast differentiation, which will deepen our understanding of oral biology and oral diseases.

## Regulation of osteoblast differentiation

### *Characteristics of osteoblasts and their progenitors*

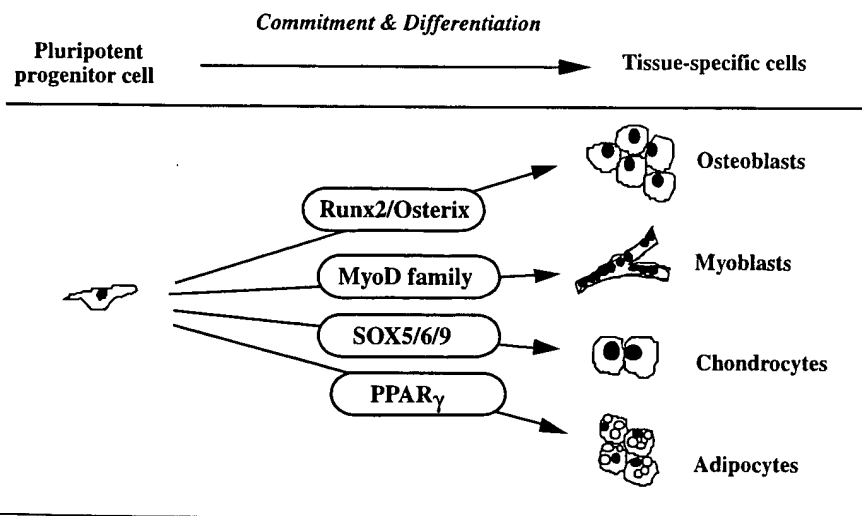
Osteoblasts are specialized cells that function in bone formation in vertebrates. Bone tissue mainly consists of hydroxyapatite crystals and various kinds of extracellular matrix proteins including type I collagen, osteocalcin, osteonectin, osteopontin, bone sialoprotein and proteoglycans (Young *et al*, 1992; Robey *et al*, 1993; Mundlos and Olsen, 1997). Most of these bone matrix proteins are secreted and deposited by polarized mature osteoblasts, which are aligned on the bone surface. The formation of hydroxyapatite crystals in osteoid is also regulated by osteoblasts. Therefore, the expression of a number of bone-related extracellular matrix proteins, high enzyme activity of alkaline phosphatase (ALP), and responses to osteotropic hormones and cytokines are believed to be major characteristics of osteoblasts.

During embryogenesis, bone tissue is formed through two independent pathways: intramembranous ossification and endochondral ossification (Karsenty, 1999; Yamaguchi, Komori and Suda, 2000). In both pathways, osteoblasts play unique roles in the bone formation. In the case of intramembranous ossification, osteoblasts are differentiated directly from mesenchymal cells in the mesenchymal condensation. On the other hand, in the endochondral ossification, mesenchymal cells differentiate into chondrocytes first and form a cartilaginous template. Then osteoblasts are differentiated from the surrounding mesenchymal cells immediately

after maturation of hypertrophic chondrocytes in the template (Chung *et al*, 1998). These developmental processes of bone and cartilage suggest that osteoblasts and chondrocytes are derived from a common progenitor cell (Figure 1). Indeed, cell cultures prepared from calvaria or bone marrow show mixed populations of osteoblasts, chondrocytes, adipocytes and skeletal muscle cells. Some clonal embryonic fibroblast cell lines differentiate into multiple phenotypes of cells in response to treatment with 5-azacytidine (Taylor and Jones, 1979). The establishment of the pluripotent cell lines from the calvaria indicated that a pluripotent progenitor cell can differentiate into tissue-specific cells such as osteoblasts, chondrocytes, adipocytes and myoblasts (Grigoriadis, Heersche and Aubin, 1988, 1990; Yamaguchi and Kahn, 1991). The progenitor cells may acquire a tissue-specific phenotype concomitantly with losing their pluripotency under the control of various stimulants. Tissue-specific transcription factors regulate the differentiation of tissue-specific cells from the progenitor cells (Figure 1).

### *Discovery of BMPs*

In 1965, Urist (1965) found that demineralized bone matrix contains a unique activity that induces ectopic bone when the matrix is implanted into muscular tissue. This activity was named 'BMP'. Subsequently, cDNAs encoding several active proteins for ectopic bone formation were isolated, and the proteins were eventually renamed 'BMPs' (Wozney *et al*, 1988). More than 15 genes of BMPs have been identified in vertebrates, and several recombinant BMP proteins have been shown to induce ectopic bone formation (Kingsley, 1994, 2001; Hogan, 1996; Wozney and Rosen, 1998; Reddi, 2001). Bone-inducing activity is unique to BMPs among the growth factors. It is believed that osteoblasts are cells responsible for the secretion and deposition of BMPs into the extracellular matrix during bone formation. BMPs, except BMP-1, belong to the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, members of which are known to regulate the proliferation, differentiation and death of cells in various tissues (Hogan,



**Figure 1** A schematic model for differentiation of a mesenchymal progenitor cell into tissue-specific cells. A pluripotent progenitor cell originated from undifferentiated mesenchyme can differentiate into several kinds of tissue-specific cells such as osteoblasts, myoblasts, adipocytes and chondrocytes. Each differentiation pathway seems to be regulated by tissue-specific transcription factors: Runx2/Osterix, MyoD family, PPAR $\gamma$  and SOX5/SOX6/SOX9, respectively

1996; Massague, 2000; Miyazono, ten Dijke and Heldin, 2000; Wrana, 2000). BMPs are also involved in the organogenesis of both hard and soft tissues (Kingsley, 1994, 2001; Hogan, 1996). Although TGF- $\beta$  superfamily members have significant homology with each other, neither TGF- $\beta$  nor activin/inhibin induces ectopic bone formation (Sampath, Muthukumaran and Reddi, 1987). BMPs are the only growth factors known at present to induce the whole process of ectopic bone formation in vertebrates.

#### *Role of BMPs in skeletal development in vivo*

The unique activity of BMPs suggests that they regulate osteoblast and chondrocyte differentiation during skeletal development. Identification of skeletal abnormalities in animals and patients with mutations in the BMP genes has confirmed this hypothesis. The first such example was the case of BMP-5 in mice (Kingsley *et al*, 1992). The mutant mouse 'short ear' has a defect in a gene required for normal growth and patterning of skeletal structures, and for repair of bone fractures in adults. Kingsley *et al* (1992) showed that the short ear region encodes BMP-5, which is deleted or rearranged in several independent mutations at the short ear locus. Storm *et al* (1994) reported that mutations in Gdf5, another member of the TGF- $\beta$  superfamily, are responsible for skeletal alterations in brachypodism (bp) mice, which are characterized by skeletal abnormalities restricted to the limbs and limb joints. The human homolog of Gdf5, CDMP-1, has also been identified as a gene associated with a recessive chondrodysplasia, Hunter-Thompson type, which has a phenotype similar to that of bp mice (Thomas *et al*, 1996). Another mutation of CDMP-1 causes a chondrodysplasia of Grebe type, an autosomal recessive disorder characterized by more severe limb shortening and dysmorphogenesis than the Hunter-Thompson type (Thomas *et al*, 1997). In these patients, the mutated CDMP-1 protein shows a dominant-negative effect by preventing the secretion of other BMP members (Thomas *et al*, 1997). It has been suggested that overexpression of BMP-4 mRNA in human lymphocytes is associated with fibrodysplasia ossificans progressiva, a heritable disorder of connective tissue characterized by postnatal formation of ectopic bone in muscular tissues (Shafritz *et al*, 1996).

Other BMP-deficient mice have also been created, although some of them died at stages too early in development to examine their skeletal phenotypes. BMP-7-deficient mice have skeletal patterning defects restricted to the rib cage, skull and hindlimbs (Dudley, Lyons and Robertson, 1995; Luo *et al*, 1995). Homozygous mutant mice carrying a targeted deletion of Gdf11 (also called BMP-11) exhibit anteriorly directed homeotic transformations throughout the axial skeleton and posterior displacement of the hindlimbs (McPherson, Lawler and Lee, 1999). The skeleton of BMP-6 null mice is indistinguishable from that of wild-type mice, suggesting that BMP-2 may functionally compensate in BMP-6-null mice (Solloway *et al*, 1998). BMP-4/7 double heterozygotes develop minor defects in the rib cage and the distal parts of limbs (Katagiri *et al*, 1998).

These findings clearly indicate that BMPs are key regulators of the differentiation of osteoblasts and chondrocytes during skeletal development. However, it is still unclear whether BMPs are involved in bone and cartilage formation after birth. Interestingly, BMP-3-null mice have twice as much trabecular bone after birth as wild-type littermates, suggesting that BMP-3 is a negative determinant of bone density (Daluiski *et al*, 2001).

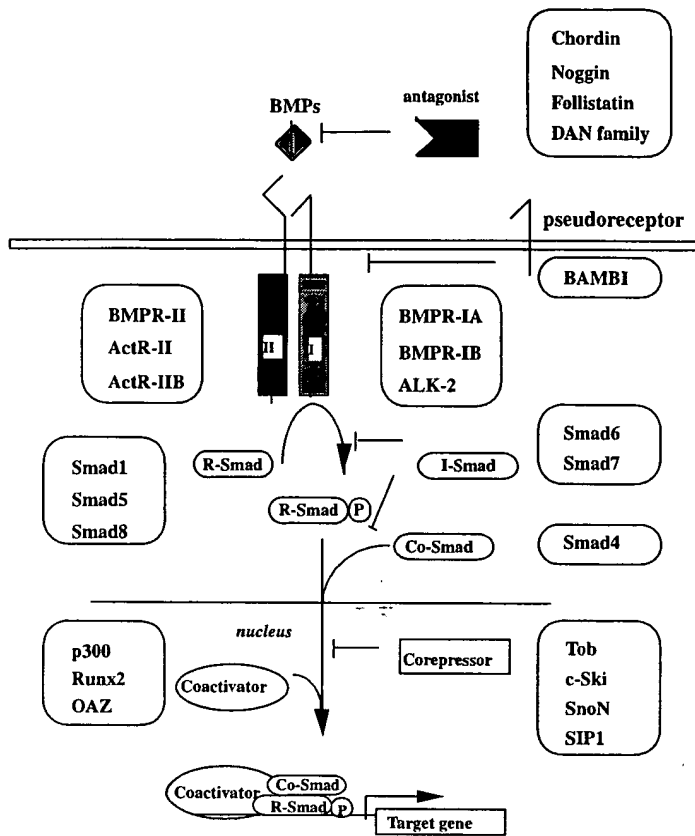
#### *Role of BMPs in osteoblast differentiation in vitro*

In order to examine the molecular mechanism of the ectopic bone-induction, the biological effects of recombinant BMP proteins on osteoblast differentiation have been studied *in vitro* using cell lines and primary cells. In cultures of osteoblast lineage cells various BMPs enhance the expression of ALP, parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor, type I collagen, and osteocalcin (Yamaguchi *et al*, 2000). Furthermore, BMPs stimulated the formation of mineralized bone-like nodules (Yamashita *et al*, 1996). BMPs also induced osteoblast differentiation in some other types of cells in culture. C3H10T1/2 clone 8 (10T1/2), a cell line established from a C3H mouse embryo, differentiates into myoblasts, adipocytes and chondrocytes in the presence 5-azacytidine (Taylor and Jones, 1979). We and others showed that BMP-2 and BMP-4 induce osteoblast differentiation of 10T1/2 cells (Katagiri *et al*, 1990; Ahrens *et al*, 1993; Wang *et al*, 1993). BMPs also stimulate osteoblast differentiation of other pluripotent cell lines (Yamaguchi *et al*, 1991; Rosen *et al*, 1994).

Bone morphogenetic proteins were originally identified as an activity that induces an ectopic bone formation in muscular tissue, suggesting that BMPs regulate the pathway of differentiation of myogenic cells. To examine this possibility, we used a mouse myoblast cell line, C2C12. We found that BMP-2 inhibited myogenic differentiation of C2C12 myoblasts, and converted their differentiation pathway into that of osteoblasts (Katagiri *et al*, 1994). TGF- $\beta$ 1 also inhibited myogenic differentiation of C2C12 cells, but failed to induce osteoblast differentiation of the cells (Katagiri *et al*, 1994). Similar effects of BMPs were observed in primary myoblasts and other myogenic cell lines in culture (Katagiri *et al*, 1994; unpublished observations). It has also been reported that the combination of BMP-2 gene transfer by adenoviruses and orthotopic muscle grafting in rats resulted in the successful ossification of almost the whole grafted muscle (Gonda *et al*, 2000). C2C12 cells are believed to have been derived from satellite cells of muscular tissue (Yaffe and Saxel, 1977; Blau, Chiu and Webster, 1983). Satellite cells are a potential source of regenerating myoblasts *in vivo*. These results suggest that satellite cells in muscular tissue are potential progenitors which can differentiate into osteoblasts in response to BMPs.

#### *BMP receptors*

Signaling by TGF- $\beta$  superfamily members, including BMPs, is basically initiated upon their binding to



**Figure 2** A schematic model for BMP signal transduction. Signaling of BMPs is initiated upon their binding to type I and II transmembrane receptors, which are serine/threonine kinases. The type I receptors phosphorylate Smad1/Smad5/Smad8 form a complex with Smad4. The complex moves into the nucleus and associates there with other DNA-binding proteins, and this large complex recognizes specific DNA motifs, and regulates the transcription of the target genes. This signaling pathway is regulated by a number of factors at multiple steps

the transmembrane receptors (Figure 2). Two types of the transmembrane serine/threonine kinase receptors, types I and II, are required for the signal transduction. The kinase activity of the ligand-bound type II receptor phosphorylates the GS domain of the type I receptor kinase. Substitution mutations of the type I receptor activated the downstream signal transduction without ligand-binding or phosphorylation by the type II receptor (Wieser, Wrana and Massague, 1995). Overexpression of the constitutively active BMP type I receptors, such as BMPR-IA, BMPR-IB and ALK-2, induced osteoblast differentiation in some types of cells (Akiyama *et al*, 1997; Chen *et al*, 1998; Fujii *et al*, 1999; Aoki *et al*, 2001). In contrast, when kinase domain-truncated BMP type I receptors were overexpressed in progenitor cells, the cells failed to differentiate into osteoblasts even in the presence of BMPs (Namiki *et al*, 1997; Chen *et al*, 1998). BMPR-IA is also involved in adipogenic differentiation of calvaria-derived cells (Chen *et al*, 1998). The binding of BMPs to receptors is regulated at multiple steps (Figure 2). The BMP type II receptors increase the ligand binding affinity of the type I receptors (Rosenzweig *et al*, 1995; Beppu *et al*, 1997). BAMBI, a pseudoreceptor of the TGF- $\beta$  family, stably associates with type I receptors and inhibits BMP-, TGF- $\beta$ - and activin- induced signals by preventing the formation of receptor complexes. (Onichtchouk *et al*, 1999). Several secreted proteins such as chordin, noggin, follistatin and DAN family members bind to BMPs and act as antagonists that inhibit the binding of

the BMPs to receptors (Piccolo *et al*, 1996, 1999; Zimmerman, De Jesus-Escobar and Harland, 1996; Hsu *et al*, 1998; Iemura *et al*, 1998). Defects in joint development are observed in noggin-deficient mice (Brunet *et al*, 1998). BMP-1 acts as a protease that releases the carboxy-terminal propeptide from type I collagen (Kessler *et al*, 1996). Interestingly, a *Xenopus* homolog of BMP-1 releases active BMPs from the chordin-BMP complex by cleaving chordin (Piccolo *et al*, 1997).

#### Role of Smads in osteoblast differentiation

Smad transcription factors are substrates of the activated type I receptor kinases in the cytoplasm. The phosphorylated Smad proteins move into the nucleus, bind to the regulatory regions of target genes, and regulate their transcription. Thus, Smad proteins are key molecules in the transduction of signals from the cell membrane to the nucleus (Sakou, 1998; Miyazono, 1999; Massague, 2000; Massague and Chen, 2000; Wrana, 2000; Shi, 2001). So far nine Smad proteins have been identified in vertebrates. They are classified into three subgroups, R-Smad, Co-Smad and I-Smad, according to their structure and function. The R-Smads consist of Smad1, Smad2, Smad3, Smad5 and Smad8. They are directly phosphorylated by the type I receptors at the carboxy terminal SSXS motif (Kretschmar *et al*, 1997). BMP type I receptors phosphorylate Smad1, Smad5 and Smad8, while TGF- $\beta$  and activin type I receptors phosphorylate Smad2 and Smad3.

Overexpression of Smad1, Smad5 or Smad8 induces ALP activity and osteocalcin production in C2C12 and 10T1/2 cells (Yamamoto *et al*, 1997; Nishimura *et al*, 1998; Fujii *et al*, 1999; Kawai *et al*, 2000). Smad4 is one of the Co-Smads, which cooperate with all R-Smads. In contrast, both Smad6 and Smad7 inhibit signal transduction of the TGF- $\beta$  superfamily members, so they are known as I-Smads (I indicates 'inhibitory'). I-Smads appear to be involved in a negative-feedback loop of the TGF- $\beta$  superfamily signaling, because the expression of I-Smad mRNAs is rapidly induced by BMPs and TGF- $\beta$ s. Signals other than Smad-mediated ones are also activated by the BMP type I receptors (Lou *et al*, 2000; Gallea *et al*, 2001). Therefore, Smad signals are regulated positively and negatively not only by Smads but also by transcriptional activators and/or repressors (Figure 2). Recently, Yoshida *et al* (2000) reported that tob-null mice have a greater bone mass, and their orthotopic bone formation is elevated relative to that in normal mice in response to BMP-2. They also showed that tob protein negatively regulates osteoblast proliferation and differentiation by suppressing the activity of R-Smads. BMP-2 and leukemia inhibitory factor synergistically stimulated astrocyte differentiation through the formation of a complex between Smad1 and STAT3, bridged by p300 protein (Nakashima *et al*, 1999). Thus, Smads appear to regulate the target gene expression through interaction with other transcription regulators.

#### *Role of Runx2 and Osterix in osteoblast differentiation*

The establishment of cbfa1-null mice clearly indicated that this transcription factor is essential for osteoblast differentiation, because, the mutant mice have no bone tissue or osteoblasts (Komori *et al*, 1997; Otto *et al*, 1997). Cbfa1/pebp2aA/AML3/osf-2 is a mammalian homolog of the Drosophila runt, and is now called Runx2. Moreover, Runx2 has also been identified as a gene responsible for cleidocranial dysplasia (CCD), an autosomal-dominant disease with abnormalities in bones formed by intramembranous ossification (Lee *et al*, 1997; Mundlos *et al*, 1997). The null mutation of Runx2 severely affects osteoblast differentiation but causes no abnormality in the patterning of the skeleton (Komori *et al*, 1997; Otto *et al*, 1997). Osteoblasts express high levels of Runx2 *in vivo* and *in vitro*. Runx2-deficient mice lack hypertrophic chondrocytes, suggesting that Runx2 also regulates chondrocyte differentiation (Komori *et al*, 1997). However, recent studies have revealed the complex role of Runx2 in osteoblast and chondrocyte differentiation. Overexpression of Runx2 in some non-osteoblastic cells induced the expression of osteoblast-related genes (Ducy *et al*, 1997; Harada *et al*, 1999). In contrast, Runx2 overexpression in osteoblasts *in vitro* suppressed the expression of type I collagen (Tsuiji, Ito and Noda, 1998). Transgenic mice overexpressing either a dominant-negative or wild-type form of Runx2 in osteoblasts exhibited osteopenia (Ducy *et al*, 1999; Liu *et al*, 2001). Runx2 overexpression in chondrocytes *in vivo* caused acceleration of endochondral ossification in mice because of precocious

chondrocyte maturation (Takeda *et al*, 2001; Ueta *et al*, 2001). In contrast, overexpression of dominant-negative Runx2 in chondrocytes *in vivo* suppressed their maturation and delayed endochondral ossification (Ueta *et al*, 2001). Furthermore, continuous expression of wild-type Runx2 in non-hypertrophic chondrocytes partially induced mineralization of cartilage in Runx2-null mice (Takeda *et al*, 2001). However, transdifferentiation from chondrocytes into osteoblasts was not observed in these mice (Takeda *et al*, 2001). Thus, Runx2 plays intricate roles in osteoblast and chondrocyte development.

Bone morphogenetic proteins up-regulate Runx2 mRNA expression *in vitro* (Ducy *et al*, 1997; Tsuiji *et al*, 1998). Hanai *et al* (1999) showed that R-Smads interact with Runx1/Runx2/Runx3. Zhang, Yasui and Ito (2000) also reported that a truncated Runx2 identified in a CCD patient failed to interact with Smads. Runx2 cooperated with Smads to induce osteoblast differentiation of C2C12 cells (Lee *et al*, 2000; Zhang *et al*, 2000). These lines of evidence suggest that Runx2 interacts tightly with BMP signaling through Smads in osteoblast differentiation. Further studies will be necessary to reveal the precise relationship between Runx2 and transcription factors, including Smads, in the induction of osteoblast differentiation. Elucidation of the regulatory mechanism of osteoblast differentiation will provide a new approach to the treatment of oral diseases.

Recently, Nakashima *et al* (2002) identified a novel zinc finger-containing transcription factor, named Osterix, from C2C12 cells treated with BMP-2. In Osterix-null mice, no bone formation occurred although Runx2 was expressed. Interestingly, however, Osterix was not expressed in Runx2-null mice. These results suggest that Osterix acts downstream of Runx2 during bone development.

### **Regulation of osteoclast differentiation**

#### *Osteoblasts regulate osteoclastogenesis*

Development of osteoclasts proceeds within the local microenvironment of bone. A coculture system of mouse osteoblasts/stromal cells and hemopoietic cells was developed to investigate the regulatory mechanisms of osteoclast differentiation (Takahashi *et al*, 1988; Suda, Takahashi and Martin, 1992). Osteoclast-like multinucleated cells are formed in the cocultures in response to various osteotropic factors including 1,25-dihydroxy-vitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], PTH, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and interleukin 11 (IL-11). Those multinucleated cells formed in the coculture expressed major characteristics of osteoclasts such as tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts), calcitonin receptors, p60<sup>c-src</sup>, vitronectin receptors ( $\alpha$ v $\beta$ 3), and the ability to form resorption pits on bone and dentine slices. Some mouse stromal cell lines such as MC3T3-G2/PA6 and ST2 resemble calvarial osteoblasts and support osteoclastogenesis in cocultures with mouse spleen cells (Udagawa *et al*, 1989). Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is required to induce osteoclastogenesis. The target cells of osteotropic factors for inducing osteoclast

formation in the cocultures are osteoblasts/stromal cells (Udagawa *et al*, 1995; Liu *et al*, 1998; Takeda *et al*, 1999; Sakuma *et al*, 2000). Therefore, we have proposed that osteoblasts/stromal cells induce osteoclast differentiation factor (ODF) as a membrane-associated cytokine in response to various osteotropic factors (Suda *et al*, 1992). Osteoclast progenitors recognize ODF through cell-to-cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts.

A method for obtaining highly purified osteoclasts from cocultures was established to investigate the role of osteoblasts/stromal cells in osteoclast function (Jimi *et al*, 1996; Suda *et al*, 1997). Purified osteoclasts cultured on dentine slices failed to form resorption pits. The resorptive capability of the purified osteoclasts was restored when osteoblasts/stromal cells were added to the purified osteoclasts. Cell-to-cell contact between osteoblasts/stromal cells and osteoclasts was required for inducing the pit-forming activity of osteoclasts (Jimi *et al*, 1996). Thus, osteoblasts/stromal cells play essential roles in inducing osteoclast function.

#### *Characteristics of osteoclast progenitors*

Several lines of evidence indicate that osteoclast progenitors are hemopoietic cells of the monocyte/macrophage lineage. Osteopetrotic op/op mice cannot produce functionally active macrophage colony-stimulating factor (M-CSF, also called CSF-1) because of an insertion of an extra thymidine in the coding region of the M-CSF gene (Yoshida *et al*, 1990). Experiments using the op/op mouse model have established that M-CSF produced by osteoblasts/stromal cells is a crucial factor for osteoclast formation. Administration of M-CSF to op/op mice restored impaired bone resorption (Felix, Cecchini and Fleisch, 1990; Kodama *et al*, 1991). Osteoclast progenitors in the spleen obtained from op/op mice differentiated into osteoclasts in cocultures with normal osteoblasts (Takahashi *et al*, 1991). However, calvarial osteoblasts prepared from op/op mice failed to support osteoclast development in cocultures with normal spleen cells, and the addition of M-CSF to the cocultures induced osteoclast formation in response to  $1,25(\text{OH})_2\text{D}_3$ . These findings indicate that M-CSF produced by osteoblasts/stromal cells plays an essential role in osteoclast development. Mouse peripheral blood mononuclear cells and alveolar macrophages differentiated into osteoclasts in coculture with ST2 cells, a supportive stromal cell line (Udagawa *et al*, 1990). The results of disruption of the PU.1 gene in mice also supported the monocyte/macrophage origin of osteoclasts (Tondravi *et al*, 1997). PU.1 is a myeloid- and B-cell-specific transcription factor, and PU.1(-/-) mice were found to be osteopetrotic. The development of both osteoclasts and macrophages was arrested in PU.1(-/-) mice, suggesting that this transcription factor regulates the initial stage of myeloid differentiation.

#### *Discovery of new TNF receptor-ligand family members involved in osteoclastogenesis*

The recent discovery of new members of the TNF receptor-ligand family has clarified the precise mechanism

by which osteoblasts/stromal cells regulate osteoclast differentiation and function. Simonet *et al* (1997) cloned a new member of the tumor necrosis factor (TNF) receptor family, termed osteoprotegerin (OPG), in an expressed sequence tag cDNA project. OPG lacks a transmembrane domain and represents a secreted TNF receptor member. Hepatic expression of OPG in transgenic mice results in osteopetrosis. Tsuda *et al* (1997) independently isolated a novel protein termed osteoclastogenesis inhibitory factor (OCIF) from the conditioned medium of human fibroblast cultures. The sequence of the cDNA for OCIF was identical to that of the cDNA for OPG. OPG strongly inhibited osteoclast formation induced by  $1,25(\text{OH})_2\text{D}_3$ , PTH,  $\text{PGE}_2$  or IL-11 in cocultures. Using OPG as a probe, a cDNA with an open reading frame encoding 316 amino acid residues was cloned from an expression library of ST2 cells (Yasuda *et al*, 1998). The OPG-binding molecule was a type II transmembrane protein of the TNF ligand family, and its expression in osteoblasts/stromal cells was up-regulated by osteotropic factors including  $1,25(\text{OH})_2\text{D}_3$ ,  $\text{PGE}_2$ , PTH and IL-11. A soluble form of this OPG-binding molecule together with M-CSF induced osteoclast formation from spleen cells in the absence of osteoblasts/stromal cells, and this osteoclast formation was completely inhibited by adding OPG. Thus, the OPG-binding molecule satisfied the major criteria of ODF, and therefore this molecule was renamed ODF (Yasuda *et al*, 1998). Lacey *et al* (1998) also cloned a ligand for OPG (OPGL), and it was found that OPGL was identical to ODF. Molecular cloning of ODF/OPGL demonstrated that it is identical to TRANCE (TNF-related activation-induced cytokine) (Wong *et al*, 1997) and receptor activator of nuclear factor  $\kappa\text{B}$  ligand (RANKL) (Anderson *et al*, 1997), which had been independently identified by other research groups. TRANCE was cloned during a search for apoptosis-regulatory genes in mouse T cell hybridomas. TRANCE induced activation of c-Jun N-terminal kinase (JNK) in T lymphocytes and inhibited apoptosis of mouse and human dendritic cells (Wong *et al*, 1997). A new member of the TNF receptor family, termed 'RANK', was cloned from a cDNA library of human dendritic cells (Anderson *et al*, 1997). The mouse homolog was also isolated from a fetal mouse liver cDNA library. The mouse RANK cDNA encodes a type I transmembrane protein of 625 amino acid residues. Thus, ODF, OPGL, TRANCE and RANKL are different names for the same molecule, a protein which is important for the development and function of T cells, dendritic cells and osteoclasts. RANK is the transmembrane signaling receptor for ODF/OPGL/TRANCE/RANKL. OPG/OCIF is a soluble decoy receptor for ODF/OPGL/TRANCE/RANKL. The terms 'RANKL', 'RANK' and 'OPG' are used in this article in accordance with the guidelines of The American Society for Bone and Mineral Research President's Committee on Nomenclature (2000). RANKL stimulates the pit-forming activity of mature osteoclasts (Burgess *et al*, 1999; Jimi *et al*, 1999a). Human osteoclasts are also formed in cultures of human peripheral

blood mononuclear cells in the presence of RANKL and human M-CSF (Matsuzaki *et al*, 1998).

### RANKL-RANK interaction in osteoclastogenesis

The expression of RANKL in osteoblasts/stromal cells is up-regulated by osteotropic hormones and factors such as  $1,25(\text{OH})_2\text{D}_3$ , PTH,  $\text{PGE}_2$  and IL-11. Compounds that elevate intracellular calcium, such as ionomycin, cyclopiazonic acid and thapsigargin, also induced osteoclast formation in mouse cocultures of bone marrow cells and primary osteoblasts (Takami *et al*, 1997) (Figure 3). Similarly, high calcium concentrations in the culture medium induced osteoclast formation in the cocultures. Treatment of primary osteoblasts with these compounds or the medium containing high levels of calcium stimulated the expression RANKL and OPG mRNAs (Takami *et al*, 2000). These results suggest that independent signals mediated by vitamin D receptors (VDR), cAMP, gp130 and intracellular calcium induce expression of RANKL in osteoblasts/stromal cells (Figure 3).

Receptor activator of NF- $\kappa\text{B}$  ligand knockout(-/-) mice exhibit typical osteopetrosis, with total occlusion of the bone marrow space within endosteal bone (Kong *et al*, 1999). RANKL(-/-) mice lack osteoclasts but have normal osteoclast progenitors that can differentiate into functionally active osteoclasts when cocultured with normal osteoblasts/stromal cells. Like RANKL-deficient mice, RANK(-/-) mice are characterized by severe osteopetrosis (Dougall *et al*, 1999). The osteopetrosis observed in RANK(-/-) mice but not RANKL(-/-) mice is rescued by transplantation of normal bone marrow cells, indicating that RANK(-/-) mice have an intrinsic defect in osteoclast lineage cells. These data indicate that RANK is the intrinsic cell surface determinant that mediates the effects of RANKL on bone resorption. A gene mapping study showed that the gene responsible for familial expansile osteolysis and familial Paget's disease of bone mapped to the gene encoding RANK (Hughes *et al*, 2000). This finding confirms that

RANK is involved in osteoclast differentiation and activation in humans as well.

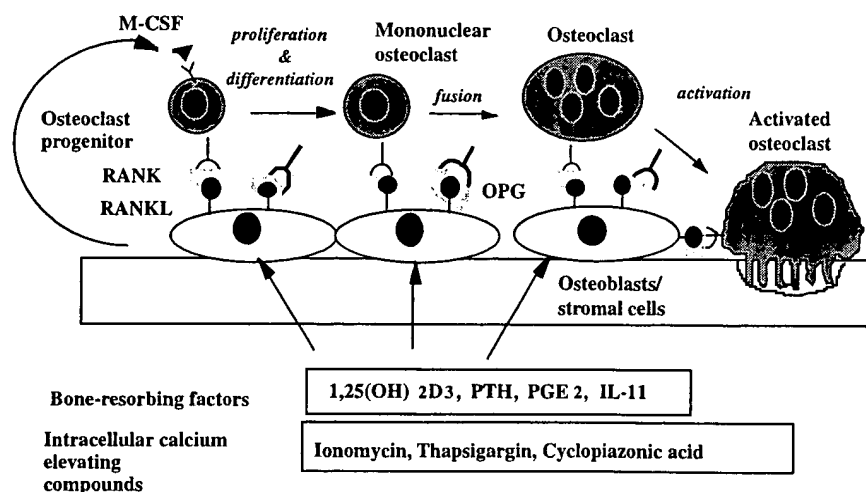
Activation of NF- $\kappa\text{B}$  and JNK through the RANK-mediated signaling system appears to be involved in the differentiation and activation of osteoclasts. The cytoplasmic tail of RANK interacts with TNF-associated factor 1 (TRAF1), TRAF2, TRAF3, TRAF5 and TRAF6 (Darnay *et al*, 1998; Galibert *et al*, 1998; Wong *et al*, 1998; Darnay *et al*, 1999; Kim *et al*, 1999). TRAF6-mediated signals appear to be important for osteoclast differentiation and function, because TRAF6(-/-) mice develop osteopetrosis with defects in bone resorption and tooth eruption (Lomaga *et al*, 1999; Naito *et al*, 1999). Mice deficient in both the p50 and p52 subunits of NF- $\kappa\text{B}$  develop severe osteopetrosis (Franzoso *et al*, 1997; Iotsova *et al*, 1997). The osteopetrotic phenotype was rescued by bone marrow transplantation, indicating that the osteoclast progenitors are inactive in the double-knockout mice. RANKL-induced activation of NF- $\kappa\text{B}$  in osteoclast progenitors seems to play a crucial role in osteoclast differentiation. Mice lacking c-Fos also develop osteopetrosis because of an early block of differentiation in the osteoclast lineage (Wang *et al*, 1992; Grigoriadis *et al*, 1994). The dimeric transcription factor activator protein-1 (AP-1) is composed of mainly Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) and Jun proteins (c-Jun, JunB and JunD). These results suggest that AP-1 appears to act downstream of RANK-mediated signals.

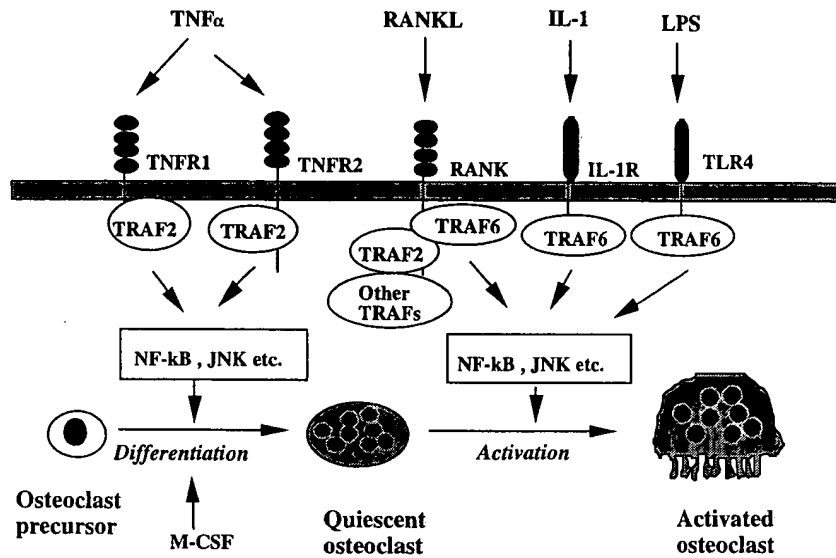
### Role of inflammatory cytokines in osteoclastogenesis

Since the discovery of the RANKL-RANK signaling system, RANKL has been regarded as the sole factor responsible for inducing osteoclast differentiation. However, recent findings indicate that inflammatory cytokines and LPS are directly involved in osteoclast differentiation and function (Figure 4).

Interleukin-1 directly stimulates osteoclast function through the IL-1 type 1 receptor expressed by osteoclasts (Jimi *et al*, 1999b). The pit-forming activity of

**Figure 3** A schematic representation of osteoclast differentiation and function supported by osteoblasts/stromal cells. Osteotropic factors such as  $1,25(\text{OH})_2\text{D}_3$ , PTH,  $\text{PGE}_2$  and IL-11 stimulate the expression of RANKL as a membrane associated factor in osteoblasts/stromal cells. Compounds that elevate intracellular calcium, such as ionomycin, cyclopiazonic acid and thapsigargin, also induce RANKL expression in osteoblasts/stromal cells. Osteoclast progenitors of the monocyte-macrophage lineage recognize RANKL expressed by osteoblasts/stromal cells through cell-to-cell interaction, and differentiate into osteoclasts. M-CSF produced by osteoblasts/stromal cells is another essential factor for osteoclast differentiation. RANKL expressed by osteoblasts/stromal cells also stimulates osteoclast function through cell-to-cell interaction





**Figure 4** Schematic representation of ligand-receptor systems in osteoclast differentiation and function regulated by TNF $\alpha$ , RANKL, IL-1 and LPS. TNF $\alpha$  and RANKL independently stimulate osteoclast differentiation via TNFR1 and TNFR2, and RANK expressed by osteoclast precursors, respectively. M-CSF is a common factor required for TNF $\alpha$ - and RANKL-induced osteoclast differentiation. TRAF2 and other TRAFs may transduce signals for the differentiation of osteoclasts. The activation of osteoclasts is induced by RANKL, IL-1 and LPS through RANK, type 1 IL-1 receptor and TLR4, respectively. TRAF6 appears to act as a common signal transducer in osteoclast activation induced by RANK, IL-1 and LPS. Signal transduction cascades such as NF- $\kappa$ B and JNK activation may be involved in the differentiation and activation of osteoclasts

osteoclasts induced by IL-1 was completely inhibited by adding IL-1 receptor antagonist (IL-1ra) but not by OPG. LPS is a cell component of Gram-negative bacteria that causes inflammatory bone loss. Recent studies identified mouse toll-like receptor 4 (TLR4) as the receptor for LPS (Poltorak *et al*, 1998; Hoshino *et al*, 1999; Qureshi *et al*, 1999). The cytoplasmic signaling cascade of TLR4 is similar to that of IL-1 receptors. Both receptors have been shown to use TRAF6 as a common signaling molecule. To examine the effect of LPS on the survival and fusion of osteoclasts, mononuclear osteoclasts (preosteoclasts, pOCs) were collected from a mouse coculture system and cultured in the presence or absence of LPS (Suda *et al*, 2001). Most pOCs died within 24 h in the absence of any stimulus. LPS as well as RANKL supported the survival of pOCs, and induced their fusion to form multinucleated osteoclasts. LPS-induced osteoclast formation in pOC cultures was observed even in the presence of OPG and IL-1 receptor antagonists. LPS induced pit-forming activity of pOCs in the presence of M-CSF. These findings suggest that LPS as well as IL-1 stimulates the survival and fusion of pOCs.

Recent studies have shown that TNF $\alpha$  directly stimulates the differentiation of osteoclast progenitors into osteoclasts in the presence of M-CSF (Azuma *et al*, 2000; Kobayashi *et al*, 2000). When mouse bone marrow cells were cultured with M-CSF, M-CSF-dependent bone marrow macrophages appeared within 3 days. Not only RANKL but also TNF $\alpha$  stimulates the differentiation of these macrophages into osteoclasts in the presence of M-CSF. Osteoclast formation induced by TNF $\alpha$  was inhibited by the addition of the respective antibodies against TNF receptor type I (TNFR1, p55) and TNF receptor type II (TNFR2, p75), but not by the addition of OPG. These results demonstrate that TNF $\alpha$  stimulates osteoclast differentiation through a mechanism independent of the RANKL-RANK system. It was also reported that when osteotropic factors such as 1,25(OH) $_2$ D $_3$ , PTHrP and IL-1 were administered to

RANK(-/-) mice, neither TRAP-positive cell formation nor hypercalcemia was induced (Li *et al*, 2000). In contrast, administration of TNF $\alpha$  to RANK(-/-) mice induced TRAP-positive cells near the site of injection, although the number of TRAP-positive cells induced by TNF $\alpha$  was not large. This suggests that TNF $\alpha$  induces osteoclast differentiation in the absence of RANK-mediated signals *in vivo*. Lam *et al* (2000) also reported that a small amount of RANKL strongly enhanced osteoclast differentiation in a pure population of murine precursors in the presence of TNF $\alpha$ . These results suggest that RANKL-induced signals cross-communicate with TNF $\alpha$ -induced ones in the target cells (Figure 4). Thus, these cytokines and LPS play important roles in osteoclastic bone resorption induced by inflammatory diseases including periodontitis. Further studies will be necessary to elucidate the regulatory mechanisms of osteoclastic bone resorption induced by inflammatory cytokines and LPS.

#### *Role of TGF- $\beta$ super family members and interferon- $\gamma$ in osteoclastogenesis*

Bone is a major storage site for TGF- $\beta$  super family members such as TGF- $\beta$  and BMPs, and osteoclastic bone resorption releases these cytokines. TGF- $\beta$  has been shown to enhance osteoclast differentiation in hematopoietic cells stimulated with RANKL and M-CSF (Sells Galvin *et al*, 1999; Quinn *et al*, 2001). Fuller, Bayley and Chambers (2000a) reported that activin A also powerfully synergized with RANKL for induction of osteoclasts from their progenitors. Moreover, osteoclast formation induced by RANKL was completely abolished by soluble activin receptor type IIA or soluble TGF- $\beta$  receptor II, suggesting that activin A and TGF- $\beta$  are essential factors for osteoclastogenesis (Fuller *et al*, 2000a; b). We also showed that BMP-2 strikingly stimulated osteoclast differentiation in the presence of RANKL and M-CSF (Itoh *et al*, 2001). OPG completely inhibited osteoclast differentiation induced by RANKL and BMP-2. A soluble form of



BMP receptor type-IA also inhibited osteoclast formation in the presence of RANKL (Itoh *et al*, 2001). We found that BMP receptor type IA mRNA was expressed on not only osteoclast progenitors but also mature osteoclasts, and that BMP-2 enhanced the survival of purified osteoclasts in the presence of RANKL but not M-CSF (Itoh *et al*, 2001). Smad1 and Smad5 are involved in the BMP signals, whereas Smad2 and Smad3 in the TGF- $\beta$  signals in the target cells. However, both BMP and TGF- $\beta$  showed similar effects on osteoclast progenitors. This suggests that signaling pathways other than Smad-mediated pathways are involved in enhancement of RANKL-induced osteoclast differentiation by TGF- $\beta$  super family members. Further studies are necessary to elucidate the molecular mechanism of the crosstalk between BMPs and RANKL in osteoclastogenesis.

Bone resorption is regulated by the immune system, where T-cell expression of RANKL may contribute to pathological conditions, such as periodontitis and autoimmune arthritis. Activated T cells also produce interferon (IFN)- $\gamma$ , which strongly suppresses osteoclastogenesis by interfering with the RANKL-RANK signaling pathway. Takayanagi *et al* (2000) reported that IFN- $\gamma$  induced rapid degradation of TRAF6, which resulted in strong inhibition of the RANKL-induced activation of NF- $\kappa$ B and JNK. This inhibition of osteoclastogenesis was rescued by overexpressing TRAF6 in precursor cells, suggesting that TRAF6 is the target critical for the IFN- $\gamma$  action. These results indicate that there is crosstalk between the TNF and IFN families of cytokines, through which IFN- $\gamma$  provides a negative link between T-cell activation and bone resorption.

## Conclusion

Bone morphogenetic proteins play critical roles in osteoblast differentiation. Smad-mediated signals are essential in BMP-induced osteoblast differentiation. Runx2 and Osterix are transcription factors required for osteoblast differentiation and bone formation. RANKL-RANK interaction is absolutely necessary for osteoclast differentiation. LPS and some inflammatory cytokines such as TNF $\alpha$  and IL-1 are directly involved in osteoclast differentiation and function through a mechanism independent of RANKL-RANK interaction. TGF- $\beta$  super family members and IFN- $\gamma$  are also important regulators in osteoclastogenesis. Further studies on the regulatory mechanisms of osteoblast and osteoclast differentiation will provide novel approaches for the treatment of bone and oral diseases.

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## A Single-Dose Placebo-Controlled Study of AMG 162, a Fully Human Monoclonal Antibody to RANKL, in Postmenopausal Women

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**ABSTRACT:** The safety and bone antiresorptive effect of a single subcutaneous dose of AMG 162, a human monoclonal antibody to RANKL, was investigated in 49 postmenopausal women. AMG 162 is a potent antiresorptive agent for diseases such as osteoporosis.

**Introduction:** RANKL is an essential osteoclastic differentiation and activation factor.

**Materials and Methods:** The bone antiresorptive activity and safety of AMG 162, a fully human monoclonal antibody to RANKL, were evaluated in postmenopausal women in this randomized, double-blind, placebo-controlled, single-dose, dose escalation study. Six cohorts of eight to nine women were randomly assigned to receive a single subcutaneous injection of either AMG 162 or placebo (3:1 ratio). AMG 162 doses were 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg. Subjects were followed up to 6 months in all cohorts and 9 months in the three highest dose cohorts. Second morning void urinary N-telopeptide/creatinine (NTX; Osteomark), serum NTX, and serum bone-specific alkaline phosphatase (BALP, Ostase) were assessed as bone turnover markers.

**Results and Conclusions:** Forty-nine women were enrolled. A single subcutaneous dose of AMG 162 resulted in a dose-dependent, rapid (within 12 h), profound (up to 84%), and sustained (up to 6 months) decrease in urinary NTX. At 6 months, there was a mean change from baseline of -81% in the 3.0 mg/kg AMG 162 group compared with -10% in the placebo group; serum NTX changes were -56% and 2%, respectively. BALP levels did not decrease remarkably until after 1 month, indicating that the effect of AMG 162 is primarily antiresorptive. Intact parathyroid hormone (PTH) levels increased up to ~3-fold after 4 days in the 3.0 mg/kg dose group, but returned toward baseline with follow-up. Albumin-adjusted serum calcium did not decrease >10% on average in any group, and no subject had values below 2 mmol/liter. AMG 162 was well tolerated. No related serious adverse events occurred. No clinically meaningful laboratory changes, other than those described above, were observed. In summary, a single subcutaneous dose of AMG 162 resulted in a dose-dependent rapid and sustained decrease from baseline in bone turnover and could be an effective and convenient treatment for osteoporosis.

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**Key words:** RANKL, osteoprotegerin, osteoporosis, bone resorption, AMG 162

### INTRODUCTION

RANKL, originally identified in dendritic cells,<sup>(1)</sup> has been well documented as a critical factor in the terminal differentiation and activation of osteoclasts.<sup>(2-4)</sup> Genetic experiments have shown that mice with a disrupted *RANKL* gene have severe osteopetrosis and absence of osteoclasts.<sup>(5)</sup> As a soluble member of the TNF receptor family, osteoprotegerin (OPG) blocks bone resorption by binding to RANKL and preventing its interaction with RANK, a cell surface receptor on pre-osteoclasts and osteoclasts.<sup>(6,7)</sup> We previously showed that an Fc-OPG fusion protein is able to

effectively block bone resorption in postmenopausal women<sup>(8)</sup> and patients with breast carcinoma or multiple myeloma.<sup>(9)</sup> Since these discoveries, RANKL signaling has been implicated in many bone diseases associated with increased bone resorption, such as osteoporosis<sup>(8,10,11)</sup>, multiple myeloma,<sup>(9,12-14)</sup> bone metastasis associated with breast carcinoma<sup>(9,15-18)</sup> and prostate carcinoma,<sup>(19,20)</sup> bone pain associated with bone metastasis,<sup>(21-24)</sup> rheumatoid arthritis,<sup>(25-29)</sup> psoriatic arthritis,<sup>(30)</sup> familial expansile osteolysis,<sup>(31)</sup> Paget's disease,<sup>(32)</sup> juvenile Paget's disease,<sup>(33)</sup> giant cell tumors,<sup>(34)</sup> and periprosthetic bone loss.<sup>(35)</sup>

AMG 162, a fully human monoclonal antibody to RANKL, blocks binding of RANKL to RANK. This report describes the findings from a single-dose, placebo-controlled study with AMG 162 in postmenopausal women to determine its safety and bone antiresorptive effect.

Drs Bekker, Holloway, Rasmussen, Murphy, Martin, and DePaoli are employees and stock holders of Amgen, Inc. Dr Dunstan received corporate appointments and stock from Amgen, Inc. All other authors have no conflict of interest.

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Results from this study have been presented previously in abstract form.

## MATERIALS AND METHODS

### *Study design*

This is a single-dose, placebo-controlled, dose escalation study in healthy postmenopausal women conducted at two centers. The main objectives were to assess safety and tolerability, the effect of AMG 162 on bone turnover as measured by biochemical markers, and the pharmacokinetics of AMG 162. Subjects (eight to nine per dose cohort) were randomized in a 3:1 ratio to receive either AMG 162 or matching placebo by subcutaneous abdominal injection. AMG 162 is a fully human monoclonal antibody (IgG<sub>2</sub>) with high affinity and specificity for human RANKL. Placebo consisted of vehicle only and was indistinguishable from AMG 162 preparations. The AMG 162 doses tested were 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg. After dosing, the three lower dose cohorts were followed for 6 months and the three higher dose cohorts for 9 months because of prolonged suppression of bone turnover.

### *Study subjects*

Subjects were at least 1 year postmenopausal and were enrolled at two study centers (Lenexa, Kansas and Miami, FL, USA). They were healthy and were not allowed to be on any therapies that might significantly affect bone turnover, e.g., bisphosphonates or fluoride within 12 months, and estrogens, selective estrogen receptor modulators, calcitonin, parathyroid hormone, high doses of Vitamin D (> 1,000 IU daily), anabolic steroids, systemic glucocorticosteroids, or calcitriol within 6 months of enrollment. Subjects were also excluded if they had evidence of a disease that might influence the results, e.g., hyperparathyroidism, hyperthyroidism, hypothyroidism, rheumatoid arthritis, Paget's disease, osteomalacia, or recent fracture (within 6 months).

### *Study procedures*

Subjects received a single dose of study drug in the morning and were followed for 6 or 9 months. The following procedures were performed during the study: medical and medication history (prestudy) and physical examinations, vital signs, hematology, serum chemistry, coagulation parameters, immunoglobulins, urinalysis, T- and B-cell enumeration (CD3, CD4, CD8, CD20, and CD56), anti-AMG 162 antibodies, and ECG (prestudy and periodically during the study). The following parameters were measured to assess the effect of AMG 162 on bone metabolism: urinary N-telopeptide/creatinine (NTX; Osteomark, Seattle, WA, USA), serum NTX (Osteomark), serum bone-specific alkaline phosphatase (BALP; Tandem-R Ostase; Hybritech, San Diego, CA, USA), serum intact parathyroid hormone (iPTH; Nichols assay), serum albumin-adjusted calcium, serum phosphorus, urinary calcium/creatinine, and urinary phosphorus/creatinine. A second morning void sample was used for the urinary parameters. Predose urine and blood samples were collected on 2 separate days before dosing to establish the baseline level for urine and serum NTX and

BALP, and the average of these two assays was used as the baseline value.

Adverse events and concomitant medications were assessed at all study visits after dosing. This study was conducted in accordance with ICH guidelines and was approved by the local Institutional Review Boards.

### *Data analysis*

The data from the subjects who received placebo in all six dose cohorts were pooled. Serum albumin-adjusted calcium (mmol/liter) was calculated as serum calcium (mmol/liter) – [0.02 × albumin (g/liter)] + 0.8. Change and percent change from baseline was calculated for all subjects for the bone metabolism parameters, and the mean change and/or percent change from baseline was compared across dose groups. The mean percent changes from baseline in urinary NTX/creatinine, serum NTX, and BALP were compared at each visit between each AMG 162 dose and placebo using a linear model controlling for dose and baseline value. Comparisons were adjusted for multiple testing using Dunnett's method.

## RESULTS

### *Demographics and baseline characteristics*

In Table 1, the baseline demographics and characteristics of the study population are summarized. No marked imbalances were noted across the dose groups. The mean age ranged from 54 to 63 years, and subjects were 7–15 years postmenopause. The majority of subjects (81%) were white, 16% were Hispanic, and 3% were black. The mean body mass index (BMI) ranged from 23.6 to 29.5 kg/m<sup>2</sup> across groups. The mean baseline urinary NTX/creatinine ranged from 36 to 66 nmol/mmol, serum NTX from 12 to 17 nmol/liter, BALP from 13 to 17 µg/liter, iPTH from 4.2 to 5.6 pM, and albumin-adjusted serum calcium from 2.26 to 2.40 mmol/liter. The bone density of study subjects at baseline is unknown, because bone densitometry was not a required study procedure.

### *Bone metabolism*

After a single subcutaneous dose of AMG 162, there was a dose-dependent decrease in bone turnover as reflected by the changes observed in urinary NTX/creatinine (Fig. 1; Table 2) and serum NTX (Table 3). At the higher doses of AMG 162, decreases were observed as early as 12 h after dosing in urine NTX/creatinine (–46% in placebo and –77% in the 3.0 mg/kg AMG 162 group; see Table 2). Note that the placebo group also showed a decrease at 12 h. This was expected because of the diurnal variation of this marker<sup>(36)</sup>: the predose urine sample was taken in the morning, when the urine NTX/creatinine level is relatively high, and the 12-h sample was taken in the early evening, when the urine NTX/creatinine level is relatively low. Twenty-four hours after dosing, there was a mean decrease from baseline in urinary NTX/creatinine of 73% in the 3.0 mg/kg AMG 162 group compared with 10% in the placebo group. The maximum urinary NTX/creatinine reduction was observed at 2 weeks in the 0.01, 0.03, 0.3, and 1.0 mg/kg AMG 162 groups, 1 month in the 0.1 mg/kg AMG 162

TABLE 1. DEMOGRAPHICS AND BASELINE CHARACTERISTICS OF THE STUDY POPULATION

	Placebo (n = 12)	AMG 162 groups					
		0.01 mg/kg (n = 6)	0.03 mg/kg (n = 6)	0.1 mg/kg (n = 6)	0.3 mg/kg (n = 6)	1.0 mg/kg (n = 6)	3.0 mg/kg (n = 7)
Age (years)	54.3 (1.9)	59.7 (5.6)	59.7 (6.0)	58.8 (9.5)	63.3 (5.9)	62.8 (4.8)	58.6 (7.4)
Years since menopause*	7.4 (4.0) <sup>†</sup>	10.0 (4.5)	13.2 (3.9) <sup>‡</sup>	13.4 (10.2) <sup>§</sup>	14.8 (8.3) <sup>‡</sup>	15.3 (5.3) <sup>§</sup>	7.6 (6.5) <sup>§</sup>
Body mass index (kg/m <sup>2</sup> )	28.4 (3.8)	28.6 (5.1)	29.5 (4.0)	23.6 (1.7)	27.9 (3.1)	28.9 (5.3)	27.7 (5.7)
Urinary NTX/creatinine (nmol BCE/mmol)	65.8 (40.3)	36.1 (9.2)	43.3 (29.1)	51.4 (19.0)	54.5 (33.1)	46.1 (12.0)	56.0 (24.8)
Serum NTX (nmol/liter)	17.2 (3.9)	11.7 (2.2)	13.9 (4.9)	16.6 (4.8)	17.1 (8.2)	16.3 (4.4)	15.2 (4.3)
Serum BALP (μg/liter)	15.4 (3.6)	13.2 (3.2)	15.4 (5.7)	14.6 (3.8)	13.1 (5.6)	13.8 (2.6)	17.0 (9.6)
Serum intact PTH (pmol/liter)	4.8 (1.6)	5.6 (2.2)	5.0 (3.1)	4.8 (1.7)	4.6 (1.2)	5.0 (1.5)	4.2 (1.2)
Serum albumin-adjusted calcium (mmol/liter)	2.34 (0.06)	2.35 (0.07)	2.40 (0.06)	2.34 (0.08)	2.40 (0.08)	2.31 (0.04)	2.26 (0.04) <sup>¶</sup>

Values are mean (SD).

\* Excludes subjects with hysterectomy and an unknown date of menopause.

<sup>†</sup> n = 11; <sup>‡</sup> n = 4; <sup>§</sup> n = 5; <sup>¶</sup> n = 6.

BCE, bone collagen equivalent.

Reference ranges: NTX/creatinine, 14–76 nmol BCE/mmol; serum NTX, 8.7–19.8 nmol/liter; BALP, 3.9–15.1 μg/liter; intact PTH, 1.1–6.9 pmol/liter; serum albumin-adjusted calcium, 2.13–2.58 mmol/liter.

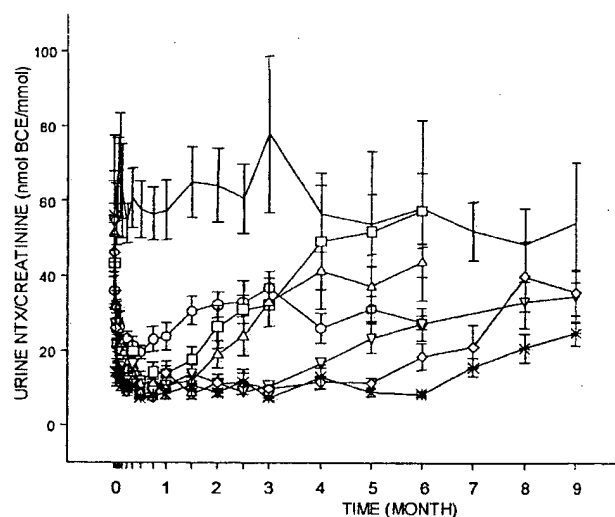


FIG. 1. The effect of AMG 162 treatment on bone resorption as reflected by changes in a second morning void urinary NTX/creatinine (nmol BCE/mmol creatinine) over time. Data are presented as mean and SEM. Placebo, no symbol; 0.01 mg/kg AMG 162, ○; 0.03 mg/kg AMG 162, □; 0.1 mg/kg AMG 162, △; 0.3 mg/kg AMG 162, ▽; 1.0 mg/kg AMG 162, \*; 3.0 mg/kg AMG 162, \*.

group, and 3 months in the 3.0 mg/kg AMG 162 group (84%). The pharmacodynamic response to AMG 162, as measured by urinary NTX/creatinine, was consistently observed across all subjects in the higher dose groups. For example, at 6 months, the range of percent changes observed was –37% to –79% in the 1.0 mg/kg group and –56% to –94% in the 3.0 mg/kg group compared with –48 to +62% in the placebo group. There was a mean change of –9% (median, –10%) in the placebo group at 9 months compared with –51% in the 3.0 mg/kg AMG 162 group (not different statistically). The treatment effect of AMG 162 was reversible, as indicated by a return toward baseline levels at ~2 months in the 0.01 and 0.03 mg/kg AMG 162 groups, 4 months in the 0.1 mg/kg group, 6 months in the

0.3 mg/kg AMG 162 group, and 9 months in the 1.0 and 3.0 mg/kg dose groups.

The serum NTX data (Table 3) confirmed the findings with urinary NTX/creatinine. The magnitude of the decreases in serum NTX was not as large as with urinary NTX. This has been observed in other studies.<sup>(37,38)</sup> The maximum mean decrease observed in any treatment group was 65% at 1 month in the 0.1 mg/kg AMG 162 group. As with urine NTX, the reversibility of the treatment effect was confirmed. At 6 months, the mean change from baseline was 2%, –43%, and –56% in the placebo, 1.0 mg/kg AMG 162, and 3.0 mg/kg AMG 162 groups, respectively, and at 9 months, –11%, –13%, and –40%, respectively, again confirming the prolonged antiresorptive effect at the higher AMG 162 doses. The change at 9 months in the 3.0 mg/kg AMG 162 group (–40%) was statistically significantly ( $p < 0.05$ ) different from placebo (–11%).

The BALP levels remained close to baseline levels in all groups up to ~2 weeks before showing a dose-dependent decrease (Table 4). The maximum mean change was –53% in the 3.0 mg/kg group at 5 months (compared with 8% in the placebo group; data not shown in Table 4), and there was still a mean change of –37% at 9 months (compared with 33% in the placebo group).

The albumin-adjusted serum calcium levels in the AMG 162-treated subjects show early but modest decreases from baseline, which were especially evident in the higher dose groups (Table 5). The maximum mean decrease at any time point was 10% (in the 0.3 mg/kg AMG 162 group at 14 days). None of the subjects had values below 2 mmol/liter. Note that subjects were instructed not to take calcium or vitamin D supplements during the study to ensure that hypocalcemic events would not be masked. The serum phosphorus levels also decreased in a similar manner to the serum calcium levels because of the antiresorptive effect of AMG 162 (data not shown). There were dose-dependent early increases in iPTH levels in the AMG 162 groups (Table 6). The maximum mean increase was ~3-fold in the 3.0 mg/kg group 4 days after dosing, but the mean levels

TABLE 2. CHANGE FROM BASELINE IN URINARY NTX/CREATININE (%)

Time point	Placebo (n = 12)	AMG 162 groups					
		0.01 mg/kg (n = 6)	0.03 mg/kg (n = 6)	0.1 mg/kg (n = 6)	0.3 mg/kg (n = 6)	1.0 mg/kg (n = 6)	3.0 mg/kg (n = 7)
12 hours	-46.3 (4.7)	-35.8 (7.3)	-45.7 (8.3)	-48.5 (15.1)	-39.0 (20.7)	-61.5 (7.9)	-76.8 (3.4) <sup>†</sup>
24 hours	-10.1 (7.0)	-8.6 (7.2)	-31.5 (7.6)	-31.9 (11.0)	-24.9 (26.8)	-37.8 (18.5)	-73.4 (5.1) <sup>‡</sup>
1 week	-9.2 (5.1)	-31.9 (8.1) <sup>†</sup>	-61.3 (5.3) <sup>‡</sup>	-66.1 (9.0) <sup>‡</sup>	-55.7 (24.5) <sup>‡</sup>	-79.6 (3.2) <sup>‡</sup>	-77.8 (6.3) <sup>‡</sup>
2 weeks	-6.5 (7.3)	-43.2 (7.7) <sup>‡</sup>	-65.7 (7.6) <sup>‡</sup>	-74.6 (5.6) <sup>‡</sup>	-68.5 (17.3) <sup>‡</sup>	-81.7 (2.2) <sup>‡</sup>	-83.2 (3.8) <sup>‡</sup>
1 month	-7.1 (5.7)	-34.5 (5.6) <sup>†</sup>	-60.7 (7.8) <sup>‡</sup>	-76.3 (4.6) <sup>‡</sup>	-56.8 (23.7) <sup>‡</sup>	-69.2 (6.5) <sup>‡</sup>	-82.3 (3.6) <sup>‡</sup>
2 months	0.3 (3.4)	-10.2 (4.9) <sup>*</sup>	-30.8 (14.9) <sup>‡</sup>	-58.5 (9.0) <sup>‡</sup>	-54.7 (23.0) <sup>‡</sup>	-74.3 (5.2) <sup>‡</sup>	-81.7 (3.1) <sup>‡</sup>
3 months	8.6 (8.7)	2.5 (10.1)	-15.4 (17.4)	-35.4 (7.2) <sup>*</sup>	-64.8 (15.6) <sup>‡</sup>	-77.9 (2.0) <sup>‡</sup>	-84.2 (3.4) <sup>‡</sup>
6 months	-9.9 (9.5)	-21.7 (5.1)	19.8 (17.8)	-5.8 (14.5)	-22.2 (27.3)	-58.7 (7.7) <sup>*</sup>	-80.9 (4.7) <sup>‡</sup>
9 months	-8.7 (15.3)	Not done	Not done	Not done	1.1 (36.6)	-13.4 (12.1)	-50.7 (8.3)

Values are mean (SE).

\* $p < 0.05$ , <sup>†</sup> $p < 0.01$ , <sup>‡</sup> $p < 0.001$ .

Linear model for the percent change from baseline contains independent effects for dose group and baseline urinary NTX (corrected). For each visit, comparisons between dose groups vs. placebo are adjusted using Dunnett's method.

TABLE 3. CHANGE FROM BASELINE IN SERUM NTX (%)

Time point	Placebo (n = 12)	AMG 162 groups					
		0.01 mg/kg (n = 6)	0.03 mg/kg (n = 6)	0.1 mg/kg (n = 6)	0.3 mg/kg (n = 6)	1.0 mg/kg (n = 6)	3.0 mg/kg (n = 7)
24 hours	-4.0 (5.4)	-9.1 (4.5)	-18.4 (7.8)	-29.0 (9.3) <sup>*</sup>	-29.1 (6.9) <sup>*</sup>	-32.7 (9.1) <sup>†</sup>	-48.8 (3.6) <sup>‡</sup>
4 days	-5.0 (3.3)	-25.6 (2.6) <sup>‡</sup>	-35.3 (4.7) <sup>‡</sup>	-51.1 (2.8) <sup>‡</sup>	-45.7 (5.8) <sup>‡</sup>	-53.0 (4.0) <sup>‡</sup>	-54.7 (3.6) <sup>‡</sup>
14 days	-10.2 (6.4)	-27.1 (2.9) <sup>*</sup>	-39.5 (5.7) <sup>‡</sup>	-49.5 (10.3) <sup>‡</sup>	-47.7 (8.5) <sup>‡</sup>	-59.4 (2.8) <sup>‡</sup>	-58.6 (5.1) <sup>‡</sup>
1 month	-5.6 (4.9)	-17.0 (3.1) <sup>*</sup>	-35.3 (6.8) <sup>‡</sup>	-64.7 (1.2) <sup>‡</sup>	-48.9 (9.3) <sup>‡</sup>	-50.5 (6.9) <sup>‡</sup>	-52.9 (5.4) <sup>‡</sup>
2 months	2.3 (3.8)	11.3 (9.6)	-14.0 (13.6) <sup>†</sup>	-48.1 (4.7) <sup>‡</sup>	-47.2 (8.8) <sup>‡</sup>	-58.6 (3.7) <sup>‡</sup>	-57.0 (5.1) <sup>‡</sup>
3 months	11.6 (5.6)	7.3 (10.9)	-2.0 (6.5)	-27.3 (5.0) <sup>†</sup>	-48.5 (8.6) <sup>‡</sup>	-53.0 (5.9) <sup>‡</sup>	-48.5 (9.8) <sup>‡</sup>
6 months	1.8 (8.0)	7.4 (5.2)	12.7 (11.4)	-15.0 (3.6)	-16.3 (11.2)	-43.1 (7.2) <sup>‡</sup>	-56.1 (7.6) <sup>‡</sup>
9 months	-11.2 (6.7)	Not done	Not done	Not done	-18.0 (10.0)	-13.4 (5.2)	-39.6 (10.3) <sup>*</sup>

Values are mean (SE).

\* $p < 0.05$ , <sup>†</sup> $p < 0.01$ , <sup>‡</sup> $p < 0.001$ .

Linear model for the percent change from baseline contains independent effects for dose group and baseline serum NTX. For each visit, comparisons between dose groups vs. placebo are adjusted using Dunnett's method.

decreased over time. At 6 months, the mean change from baseline was 8% and 67% in the 1.0 and 3.0 mg/kg groups, respectively, compared with -3% in the placebo group. There were decreases in urinary calcium/creatinine levels in subjects receiving AMG 162, but urinary phosphorus/creatinine levels remained relatively stable compared with the placebo control group (data not shown).

#### Safety and tolerability

No related serious adverse events were reported. Two unrelated events required hospitalization: a subject with moderately severe abdominal pain with no identified underlying pathology in the 0.01 mg/kg AMG 162 group, and another with cholecystitis in the 0.1 mg/kg AMG 162 group. None of the subjects were discontinued from the study because of an adverse event. The incidence of reported infectious events was similar across groups (33% in placebo and 38% in the AMG 162 group overall, with no apparent dose-dependent increase). The subcutaneous injections were well tolerated; one subject who

received 1.0 mg/kg reported injection site pain and another who received 3.0 mg/kg had injection site rash and burning. No clinically significant changes in any other laboratory variables were noted. The mean white blood cell (WBC) count remained stable (within 10% of the mean baseline value) across all AMG 162 groups, and there were no changes associated with AMG 162 treatment in T- and B-cell counts (CD3, CD4, CD8, CD20, and CD56), coagulation parameters, and immunoglobulins. Serum from all subjects was assessed on a weekly basis through month 1, biweekly through month 3, and monthly through month 9 for anti-AMG 162 antibodies, and all tests were negative.

#### Pharmacokinetics

The SC pharmacokinetics of AMG 162 in postmenopausal women were nonlinear with dose. The serum profiles were characterized by three distinct phases (Fig. 2): (1) a prolonged absorption phase, which resulted in maximum serum concentrations that increased dispropor-



TABLE 4. CHANGE FROM BASELINE IN BALP (%)

Time point	AMG 162 groups						
	Placebo (n = 12)	0.01 mg/kg (n = 6)	0.03 mg/kg (n = 6)	0.1 mg/kg (n = 6)	0.3 mg/kg (n = 6)	1.0 mg/kg (n = 6)	3.0 mg/kg (n = 7)
1 week	5.0 (3.4)	2.8 (4.1)	9.9 (4.8)	-10.2 (10.6)	1.3 (4.2)	8.5 (4.4)	-3.2 (3.2)
2 weeks	3.4 (3.9)	7.0 (5.6)	11.8 (7.1)	-3.8 (11.2)	11.5 (6.0)	0.0 (4.5)	0.7 (3.5)
1 month	8.3 (4.4)	0.1 (7.2)	4.5 (6.0)	-3.3 (8.7)	0.3 (4.8)	-8.7 (4.9)	-10.9 (5.4)
2 months	-3.7 (5.4)	-19.5 (7.9)	-34.6 (4.0) <sup>†</sup>	-32.4 (5.5) <sup>†</sup>	-21.8 (6.3)	-27.6 (4.1)	-26.4 (6.3)
3 months	-1.8 (4.9)	-21.1 (7.0) <sup>*</sup>	-33.4 (4.0) <sup>†</sup>	-30.3 (9.1) <sup>†</sup>	-34.7 (6.5) <sup>†</sup>	-42.2 (5.5) <sup>†</sup>	-38.7 (7.0) <sup>†</sup>
6 months	3.8 (4.2)	3.7 (9.9)	-18.0 (7.6)	-14.6 (14.0)	-31.8 (9.5) <sup>†</sup>	-36.6 (7.9) <sup>*</sup>	-50.0 (4.5) <sup>†</sup>
9 months	33.2 (10.8)	Not done	Not done	Not done	-1.3 (12.2) <sup>*</sup>	-5.9 (9.4) <sup>*</sup>	-36.8 (10.3) <sup>†</sup>

Values are mean (SE).

<sup>\*</sup> $p < 0.05$ , <sup>†</sup> $p < 0.01$ , <sup>‡</sup> $p < 0.001$ .

Linear model for the percent change from baseline contains independent effects for dose group and baseline BALP. For each visit, comparisons between dose groups vs. placebo are adjusted using Dunnett's method.

TABLE 5. ALBUMIN-ADJUSTED SERUM CALCIUM (mmol/liter)

Time point	AMG 162 groups						
	Placebo (n = 12)	0.01 mg/kg (n = 6)	0.03 mg/kg (n = 6)	0.1 mg/kg (n = 6)	0.3 mg/kg (n = 6)	1.0 mg/kg (n = 6)	3.0 mg/kg (n = 7)
Baseline	2.34 (0.02)	2.35 (0.03)	2.40 (0.02)	2.34 (0.03)	2.40 (0.03)	2.31 (0.02)	2.26 (0.02)
24 hours	2.36 (0.02)	2.37 (0.03)	2.39 (0.01)	2.34 (0.03)	2.32 (0.02)	2.28 (0.02)	2.19 (0.02)
4 days	2.36 (0.02)	2.28 (0.03)	2.31 (0.03)	2.23 (0.04)	2.30 (0.03)	2.25 (0.02)	2.13 (0.03)
14 days	2.32 (0.03)	2.35 (0.01)	2.29 (0.02)	2.28 (0.04)	2.16 (0.02)	2.19 (0.04)	2.11 (0.03)
1 month	2.34 (0.03)	2.36 (0.03)	2.33 (0.03)	2.17 (0.03)	2.22 (0.03)	2.25 (0.02)	2.19 (0.02)
2 months	2.29 (0.02)	2.24 (0.02)	2.26 (0.02)	2.27 (0.04)	2.21 (0.03)	2.18 (0.02)	2.20 (0.02)
3 months	2.27 (0.01)	2.23 (0.01)	2.31 (0.01)	2.22 (0.05)	2.20 (0.03)	2.19 (0.03)	2.16 (0.03)
6 months	2.29 (0.01)	2.23 (0.02)	2.35 (0.02)	2.28 (0.02)	2.25 (0.03)	2.32 (0.03)	2.22 (0.02)
9 months	2.33 (0.04)	Not done	Not done	Not done	2.28 (0.05)	2.28 <sup>*</sup>	2.26 (0.04)

Values are mean (SE).

<sup>\*</sup> Cannot calculate SE because  $n = 1$ .

Reference range: 2.13–2.58 mmol/liter.

TABLE 6. SERUM INTACT PTH (pmol/liter)

	AMG 162 groups						
	Placebo (n = 12)	0.01 mg/kg (n = 6)	0.03 mg/kg (n = 6)	0.1 mg/kg (n = 6)	0.3 mg/kg (n = 6)	1.0 mg/kg (n = 6)	3.0 mg/kg (n = 7)
Baseline	4.80 (0.46)	5.57 (0.89)	4.99 (1.26)	4.83 (0.67)	4.60 (0.48)	5.04 (0.59)	4.23 (0.44)
24 hours	4.54 (0.26)	5.50 (0.75)	5.16 (0.85)	6.63 (0.76)	5.73 (0.82)	7.23 (1.07)	7.05 (0.96)
4 days	4.55 (0.30)	5.98 (0.83)	7.52 (2.19)	9.44 (1.85)	8.91 (2.47)	10.70 (1.79)	12.79 (2.07)
14 days	4.50 (0.35)	5.13 (0.67)	9.18 (2.18)	11.09 (1.40)	9.30 (2.10)	12.38 (4.28)	11.40 (3.16)
1 month	4.91 (0.53)	6.33 (0.72)	9.11 (2.15)	10.35 (2.07)	10.13 (3.30)	8.33 (1.29)	9.10 (1.14)
2 months	5.34 (0.51)	5.52 (0.67)	7.96 (1.91)	8.14 (0.84)	9.25 (2.40)	8.14 (1.31)	9.10 (1.97)
3 months	5.12 (0.62)	5.27 (1.01)	4.69 (0.45)	5.55 (1.08)	7.09 (0.74)	9.07 (1.99)	8.49 (1.68)
6 months	4.38 (0.34)	3.87 (0.64)	4.10 (0.44)	4.79 (0.57)	5.34 (0.58)	5.31 (0.70)	7.05 (1.09)
9 months	4.77 (1.28)	Not done	Not done	Not done	3.47 (0.87)	4.03 <sup>*</sup>	6.53 (1.54)

Values are mean (SE).

<sup>\*</sup> Cannot calculate SE because  $n = 1$ .

Reference range: 1.1–6.9 pmol/liter.

tionately greater (2.6-fold) than the increase in dose and were observed between 5 and 21 days after administration; (2) a prolonged  $\beta$ -phase, characterized by half-lives that increased with dose to a maximum of 32 days; and

(3) a more rapid terminal phase observed at concentrations  $<1000$  ng/ml with a half-life that increased from 5 to 10 days as dose increased from 0.01 to 3.0 mg/kg. Because of the nonlinear pharmacokinetics, the mean

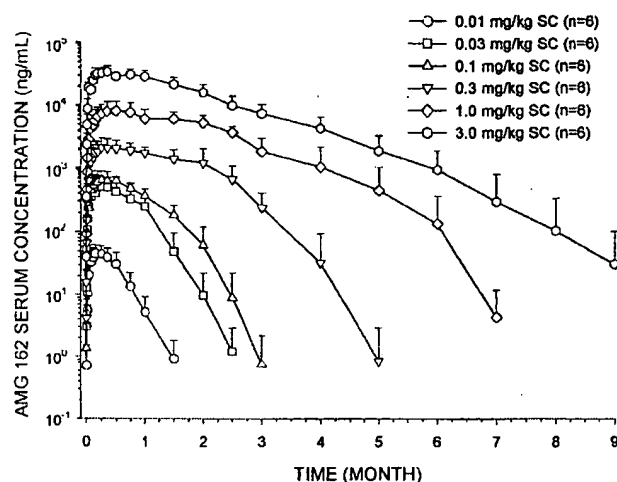


FIG. 2. The serum concentration profile of AMG 162 (ng/ml) over time. Data are presented as mean and SEM.

serum residence time (MRT) increased with dose from 12 to 46 days.

## DISCUSSION

Because RANKL plays such a pivotal role in osteoclast development and bone resorptive activity, the ability to control its interaction with RANK and thereby manage bone diseases characterized by increased osteoclastic activity, such as osteoporosis, bone metastasis, rheumatoid arthritis, Paget's disease, and others, is compelling. AMG 162, a specific fully human monoclonal antibody to RANKL, which prevents RANKL binding to RANK, has been developed and tested in this first-in-human clinical study. The results show that AMG 162 seems to specifically and profoundly inhibit osteoclastic bone resorption, as indicated by the changes observed in the biochemical markers, urinary NTX/creatinine, and serum NTX. The effect is evident within 12 h of dosing, which indicates that mature and active osteoclastic activity is inhibited almost immediately. The effect is also profound as indicated by the low NTX levels observed during the period of maximum effect, which increases as the dose was increased. Therefore, little if any osteoclastic activity remains while AMG 162 is in circulation, which is for a prolonged period based on the pharmacokinetic profile. However, the effect is reversible as indicated by a return of NTX levels when AMG 162 is cleared from the circulation.

BALP levels do not show such a rapid decrease as NTX. This was anticipated, because AMG 162 does not primarily interfere with osteoblastic activity. By interrupting terminal osteoclastic development, it reduces the activation frequency (or birth rate) of basic multicellular units, the cellular units responsible for bone turnover. Osteoclastic bone resorption on dormant bone surfaces normally initiates the bone turnover cycle, which is followed by osteoblastic bone formation.<sup>(39,40)</sup> Therefore, when the activation frequency is decreased, the bone formation rate also decreases as a result. This decrease in BALP has been observed with bisphosphonates<sup>(41-43)</sup> and raloxifene.<sup>(44)</sup>

The degree of bone turnover suppression (up to 81% in the 3.0 mg/kg dose at month 6) is at least comparable with the most potent antiresorptive agents. Alendronate, at the marketed dose of 10 mg daily, is associated with a mean decrease in urinary NTX/creatinine of 64–70%,<sup>(41,45)</sup> and at 70 mg weekly, the mean decrease reported was 56%.<sup>(41)</sup> Risedronate (5 mg daily) resulted in a mean decrease of 40–60%,<sup>(46,47)</sup> and with 35 mg weekly, 61%.<sup>(47)</sup> A mean decrease of 54–65% has been reported at month 12 with 4 mg intravenous zoledronate.<sup>(43)</sup> Raloxifene (60 mg daily) showed a mean decrease of 17%.<sup>(44)</sup>

Transient decreases in serum albumin-adjusted calcium, phosphorus, and urinary calcium/creatinine are consistent with the antiresorptive effect of AMG 162, as is the compensatory increase in PTH secretion.

Because AMG 162 is effectively acting similarly to OPG, it is of interest to compare them. The safety and efficacy of Fc-OPG fusion molecules were studied in postmenopausal women<sup>(8)</sup> and patients with breast carcinoma-associated bone metastases or multiple myeloma<sup>(9)</sup> and indicated that these molecules are effective antiresorptive agents. However, data from the study reported here indicate that AMG 162 has superior characteristics: it is more potent, showing greater decreases in bone turnover markers at lower doses, and the duration of antiresorptive effect is also longer at equivalent doses.

AMG 162 seemed to be well tolerated, and no significant safety issues have been identified. Although AMG 162 is a potent antiresorptive agent, no significant degree of hypocalcemia has been observed. It is of note that the nadir of the mean serum albumin-adjusted calcium was observed 2–8 weeks after doses of 0.3 mg/kg or higher of AMG 162, which is later than observed with the Fc-OPG fusion molecules (2–8 days after dosing<sup>(8)</sup>; data on file). This may be related to differences in the pharmacokinetics of the compounds.

Partial inhibition of early T- and B-lymphocyte development has been observed in RANKL-deficient mice.<sup>(5)</sup> There was no clinically significant effect on lymphocyte counts overall (CD3), T-cells (CD4, CD8, CD56), or B-cells (CD20) in this study, and no meaningful differences were observed among treatment groups regarding incidence of infectious events.

A potential risk with an OPG molecule is the generation of anti-OPG antibodies, which might cross-react with endogenous OPG, neutralizing its activity. Anti-OPG antibodies were observed in one subject in a phase 1 study with an Fc-OPG fusion molecule (P Bekker, unpublished observations, 2001), and although there was no apparent negative effect clinically, safety concerns could arise with chronic dosing. This prospect is avoided with AMG 162, because it does not resemble OPG structurally, and even if anti-AMG 162 antibodies were elicited, they would not be expected to cross-react with endogenous OPG. No evidence of anti-AMG 162 antibodies was observed in this study.

Another potential concern is binding of OPG to TNF-related apoptosis-inducing ligand (TRAIL,<sup>(48)</sup> a survival factor for tumor cells) and interference with a natural defense mechanism against tumorigenesis. Binding of OPG to a soluble form of TRAIL has been reported.<sup>(49)</sup> Although

OPG has a low affinity for membrane-associated TRAIL (B Boyle, unpublished observations, 2001), this could nevertheless be a potential concern at high doses of OPG. Because AMG 162 is specific for RANKL, it does not bind to TRAIL. In an in vitro competition assay with RANKL-expressing Chinese hamster ovary cells, TRAIL, TNF $\alpha$ , TNF $\beta$ , and CD40L (tested at doses up to 1  $\mu$ g/ml) were unable to compete with anti-RANKL antibody binding to RANKL, whereas this binding was competed by exogenously added human RANKL (P Kostenuik, unpublished observations, 2004). Therefore, the concern regarding AMG 162 binding to TRAIL was alleviated.

The effect of AMG 162 treatment at the bone histological level has not been assessed in this study, and conclusions regarding its safety in this regard cannot be drawn. This will require larger, long-term studies.

The results from this study indicate that AMG 162 could potentially have therapeutic applications to several bone disorders, including osteoporosis, cancer-related bone disease, rheumatoid arthritis, psoriatic arthritis, Paget's disease, osteoclastoma, prosthesis loosening, and periodontal disease. Longer-term, multiple dose studies are ongoing to assess the use of AMG 162 in modifying the course of bone disease.

Although this study was conducted at only two study centers in a small number of healthy postmenopausal subjects, it is likely that, based on results from this study, a similar bone antiresorptive effect would be observed in osteoporotic patients and those with other bone diseases. The effects of AMG 162 on bone density were not assessed in this study, but it is likely, based on the data from this study and the established link between bone resorption and bone density, that a positive effect on bone density would be observed in patients treated with AMG 162. Ongoing studies are assessing the effects of this treatment on bone density.

In summary, this randomized, single-dose, double-blind, placebo-controlled study showed that AMG 162, a fully human, high-affinity, specific anti-RANKL monoclonal antibody, is a potent, long-acting, well-tolerated bone antiresorptive agent with potential broad application in the treatment of bone disorders.

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# Bone morphogenetic proteins (BMPs): how do they function and what can they offer the clinician?

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**Abstract:** Bone Morphogenetic Proteins (BMPs) form a unique group of proteins within the Transforming Growth Factor beta (TGF- $\beta$ ) superfamily of genes and have pivotal roles in the regulation of bone induction, maintenance and repair. They act through an autocrine or paracrine mechanism by binding to cell surface receptors and initiating a sequence of downstream events that have effects on various cell types. Differentiation of osteoprogenitor mesenchymal cells and up-regulation of osteoblastic features occur under the influence of cytokines and growth factors that are expressed with the direct or indirect guidance of BMPs acting at the transcriptional level or higher. The Smads family of proteins has been identified as the downstream propagator of BMP signals, whereas hedgehog genes are possible modulators of BMP expression. The inflammatory response observed during wound repair and fracture healing, results in by-products that interact with BMPs and affect their biologic potential. Additive, negative or synergistic effects are observed when homodimeric or heterodimeric forms of BMPs interact with BMP receptors. Storage within the bone matrix allows for their involvement in the modeling/remodeling process by mediating coupling of osteoblasts and osteoclasts. Micro-environmental conditions, dose, possible carrier materials and geometrical parameters of delivery matrix are critical determinants of the

pharmacokinetics of BMP action and the biologic outcome during wound repair. Because of their osteogenic potential, BMPs are of tremendous interest as therapeutic agents for healing fractures of bones, preventing osteoporosis, treating periodontal defects and enhancing bone formation around alloplastic materials implanted in bone. (J. Oral Sci. 45, 57-73, 2003)

Key words: bone morphogenetic proteins; bone; growth factors; delivery systems; therapeutic regeneration.

## Introduction

Bone Morphogenetic Proteins (BMPs) form a unique group of proteins within the Transforming Growth Factor beta (TGF- $\beta$ ) superfamily. BMPs were first identified by Urist in 1965 when demineralized bone matrix implanted in ectopic sites in rats was found to induce bone formation (1). There is extensive evidence supporting their role as regulators of bone induction, maintenance and repair, as well as being critical determinants of the embryological development of mammalian organisms (2,3). During embryogenesis, they regulate dorsal-ventral patterning (4), establishment of embryonic body plan (4), cell apoptosis (5), differentiation of neural cells (6,7), patterning of the limb bud (5) and epithelial-mesenchymal interactions during organogenesis (8,9). The TGF- $\beta$  superfamily includes the activins/inhibins, BMPs, TGF- $\beta$ , growth and differentiation factors (GDFs), mullerian inhibiting substance, *Drosophila dpp* and *Xenopus Vg1*, amongst others (10). BMPs play a role in the differentiation, proliferation, growth inhibition and arrest of maturation

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of a wide variety of cells, depending on the cellular micro-environment and the interactions with other regulatory factors (11).

### Ligand-receptor interactions and downstream events

At their carboxy-terminal ends, all BMPs possess a region containing seven cysteine residues that is conserved among all the reported members of the TGF- $\beta$  superfamily (12). BMPs are synthesized inside the cell in a precursor form with a hydrophobic secretory leader and a pro-peptide sequences joined to the mature region. Proteolytic cleavage frees the mature region, which can then dimerize with other BMPs. Mature BMP-2 is a homodimer of two 114-peptides subunits (13). Dimeric molecules can be either homodimers, when both subunits are the same, or heterodimers, consisting of two different subunits. Structural and chemical differences between the homodimeric and heterodimeric forms may be responsible for variations of their biologic potential and binding characteristics.

BMPs initiate signaling from the cell surface when they bind to and bring together type I and type II serine-threonine kinase receptors (14,15), both of which have sub-categories (17). BMP receptors are composed of three parts: a short extracellular domain, a single membrane-spanning domain, and an intracellular domain with the active serine/threonine region (18). The 75kDa type II receptor is the primary binding site of the ligand and upon its activation, phosphorylation of the type I receptor (50-55 kDa) occurs (14,17). It is only after the type I receptor becomes phosphorylated that receptor signals are propagated to downstream substrates (9). It is believed that the type II receptor does not actually bind the ligand but rather stabilizes the type I receptor (19), or accelerates ligand binding to the type I receptor (20).

Various signaling pathways have been proposed to be activated by the ligand binding to the receptors. Recently, a novel family of proteins, the Smad family, have been identified as the downstream effectors of the phosphorylated type I receptor (21-23). More specifically, Smads 1 and 5 become phosphorylated at the carboxy-terminal serine residues and then translocate to the nucleus (24) where they interact with DNA binding proteins (25), or exhibit direct transcriptional activity (26), either as monomers or in association with Smad 4 (21). Specific Smads are expressed at different phases of the cell cycle and exhibit either an inhibitory or stimulatory function (27). Smads 6/7 have been shown to antagonize and inhibit phosphorylation of Smads 1/5 by BMP-2 (28,29). Phosphorylation of Smad1 leads to the transcriptional activation of the DPC4 gene

that expresses Smad4 and the subsequent Smad1/4 complex translocates to the nucleus (21). The lack of DNA binding motifs on Smads does not allow their direct association with genomic sequences. It has been shown that small interacting proteins are necessary in bridging molecules and helping to mediate Smads action.

Genetic screens in *Drosophila* revealed that the gene *schurri* becomes up-regulated after BMP binds to their receptors, leading to the formation of an active transcription factor similar to zinc finger proteins in mammals (30). In addition, the protein kinase TAK1 has been isolated and shown to transduce signals downstream following BMP binding and function as a mitogen-activated protein kinase (31). Overall, BMPs regulate cell function at the transcriptional level or higher (32), by increasing the rate of transcription and/or stabilizing the mRNA (33). Moreover, the large number (2,500/cell) of BMP receptors (6) and their variability (16,29) allows heteromeric complexes to be formed with different signaling potential (16) capable of inducing various responsive cascades when binding to the same ligand (29,34).

Another line of evidence indicates the involvement of the Ras/Raf pathway in the downstream response to BMPs (35). According to this model, the signal is transmitted through Ras to Raf and, subsequently, activates other transcription factors such as AP-1 and GATA-2. BMP-2 up-regulates Id (inhibitor of differentiation) gene expression in osteoblastic cells and promotes their specific phenotypic expression (32). BMP-2 has an effect on cell-matrix interactions by suppressing the expression of the  $\alpha 3$  integrin subunit at the mRNA level (36), suggesting that BMP action, in part, may be exerted through altered cell adhesion to the extracellular matrix (ECM), modified cell migration and differentiation (37,38). In another report, cadherin expression in osteoblasts was unaffected by BMP-2 suggesting that the latter does not play a role in cell-cell interactions (39).

The family of Hedgehog genes (*Sonic hedgehog*, (*Shh*), *Desert hedgehog*, (*Dhh*) and *Indian hedgehog*, (*Ihh*)) present a striking correlation with expression of BMP genes in the mouse embryo (40) and act as possible modulators of BMP expression (41). In addition, growth factors such as TGF- $\beta$ 1 exert a negative regulation on BMP-2 at the transcription level (42) and retinoic acid receptors also affect BMP expression (43). Noggin, chordin, cerberus, dan and gremlin are some of the molecules that have recently been found to regulate BMP expression and modulate their role in eliciting various biologic responses (44). A number of BMP-binding proteins (lipovitellin 1, Ep45) (45) and antagonist molecules (noggin) (46), affect and control the presence of growth

factors thus defining various concentration gradients, which has been shown to play a role in cytodifferentiation. There are several reports on the associated action of BMPs and noggin (47,48) suggesting that transcription factors from the *fos* family are downstream targets of noggin-related calcium regulation (47). These transcription products in turn limit the stimulatory action of BMPs on osteoblasts and thus act as autocrine regulators (49).

In summary, the variety of BMP receptors and the numerous pathways they regulate, together with the fact that BMPs exist in the form of homodimers, suggest that they may evoke synergistic, negative or additive net effects. This draws attention to the complexity of the signaling cascade and the variability of downstream responses to BMP-receptor signaling.

### BMPs in bone formation

Bone formation can take place *via* a direct (intramembranous) or an indirect (endochondral) process. Intramembranous ossification occurs during embryonic development of the cranial vault bones by the direct transformation of mesenchymal cells into osteoblasts. Endochondral ossification, which is the process by which long bones develop, involves the formation of an intermediate cartilaginous anlagen that eventually becomes ossified and contains all the cellular components of mature bone (50). In both mechanisms, the induction of bone and cartilage occurs through an epithelial-mesenchymal interaction (51) that initiates specific cell differentiation and leads to precursor cells of the osteoblastic or chondroblastic lineage.

The development of cartilage and bone from mesenchyme, is characterized initially by a condensation of mesenchymal cells (52). This condensation can occur in two ways: either by cells moving toward a central focal point or by a localized region of increased proliferation. Direct cell-to-cell contact, diffusible molecules produced by the signaling cells, or matrix mediated interactions can result in a cellular mass of increased proliferative activity (53-55). An early step in the endochondral bone formation process is the condensation of mesenchymal cells into discrete pre-cartilaginous nodules. Chondrogenic cells become hypertrophic and pass into a bio-synthetically active state that involves mineral deposition in the cartilaginous matrix. The hypertrophic chondrocytes will then secrete chemotactic agents that both attract and direct the invasion of the sites by blood vessels. The chondrocytes eventually die and their matrix is partially destroyed during vascular invasion, which is when osteoblasts appear. Initially, osteoid will be deposited and remodeling will finally produce functional bone tissue (56).

Glucocorticoids mediate their action on osteoblasts through BMPs (57) and retinoic acid, a derivative of vitamin A, is a possible modulator of BMP expression (58). Cell adhesion molecules such as laminins (59), neural cell adhesion molecules (N-CAM) (60), and integrins (36) are known to interact with BMPs and other growth factors and have been localized at the areas of initial mesenchymal condensation (60,61). Related to this intra-molecular interaction are the chemotactic properties of BMPs. Depending on their concentration gradient, BMPs can attract various types of cells (19,62) and act as chemotactic, mitogenic or differentiating agents (63,64). BMPs may affect proliferation of cartilage-forming and bone-forming cells and can induce differentiation of mesenchymal progenitor cells into various cell types, including chondroblasts and osteoblasts (19,65). The latter property suggests that BMPs may be able to influence both the endochondral bone induction pathway and direct bone formation. In ectopic bone formation, associated with implantation of BMPs, the sequence of events recapitulates the process of bone formation that is observed during embryonic long bone development and many of the BMP properties can be extrapolated from there.

One of the most difficult subjects to study with regard to the *in vivo* response to BMPs is the characterization of the responding cell population. The wide spectrum of cells that are sensitive to BMP action includes fibroblasts (61), mesenchymal connective tissue cells (66), muscle derived connective tissue cells (67), the astroglial lineage (68) and many more (61). Bone marrow stromal cells form an important source of mesenchymal pluripotential progenitors that are capable of differentiating into various cell lineages under the appropriate conditions. Demineralized bone matrix, dexamethazone, beta glycerophosphate, vitamin D and BMP-1 have been shown to stimulate bone marrow stromal cells to take on an osteoblastic phenotype (69, 70). Bone marrow mesenchymal cells have the potential to differentiate along the osteoblastic and adipocytic lineages. Studies have demonstrated a concentration specific response with lower doses of BMPs inducing the adipocyte lineage and higher doses eliciting a chondrogenic/osteoblastic response (71). Treatment with rhBMP-2 protects and enhances cell commitment towards the osteoblastic phenotype (72,73). Osteoblasts and chondroblasts originate from a common precursor which is a bipotential mesenchymal progenitor called osteo-chondroprogenitor (74) or skeletoblast (75-78). Osteoprogenitors can be classified as either determined or inducible, based on their need for additional signals in order to differentiate (58,79). This difference is important as it reflects the variation between cell commitment, when the fate of cells is



programmed, and cell differentiation, when the fate of cells is expressed due to the permissive signals of the micro-environment. The answer to this debate is not clear yet but it is obvious that some form of control over phenotypic expression must occur to ensure that the tissues develop in a coordinated fashion in the appropriate places and amounts. (80). Evidence supports the hypothesis that BMPs act on the skeletal progenitor cells and induce the differentiation of both the osteoblast (65,71,81-83) and chondroblast (56,60,84,85).

Factors affecting BMPs bone inductive ability are amounts, qualitative composition, possible presence of inhibitors, correct processing and storage (86). In addition, the dose, concentration and time of BMP action are important parameters of the inductive outcome (64,87). Low concentrations of BMP-2 (50ng/ml) up-regulated the expression of the collagen II gene whereas higher concentrations (100-400ng/ml) inhibited collagen II expression in chondrocyte cell lines and increased osteocalcin (OC) expression (72). These results clearly show that chondrocytes are able to express osteoblastic features. It is more realistic to assume that BMPs induce cytodifferentiation along those lineages when permissive conditions for each cell type exist. Stability or structural integrity that allows blood vessels to grow (88), micro-environmental conditions that affect oxygen tension (89), and geometrical/architectural characteristics of the ECM affecting the cytoskeleton (90) through membrane receptors are critical factors for cytodifferentiation. Differences in the partial pressure oxygen and in the amount of mesenchymal cells present in intramuscular and subcutaneous sites are responsible for the lower dose of BMP needed to induce ectopic bone formation in the former site (91).

Treatment with cytochalasin D disrupts the cytoskeleton, making cells spherical in shape and resulting in high concentrations of endogenous soluble and matrix factors, thus promoting the chondrogenic phenotype (92). Reduced serum, high cell density, and type I collagen have been described as necessary parameters for chondrogenic differentiation (93). Cells at high density become attached to type I collagen leading to cellular condensation that promotes the chondrogenic phenotype. Further differentiation into hypertrophic chondrocytes and mineralization does not require BMP-2 but it is dependent on the presence of ascorbic acid and serum factors (94,95). Depending on the dose and the coordinated action of other cytokines (100,101), BMP-2 plays a regulatory role for the sequential progression of chondrocytes through their maturation (96,97), with development of hemopoietic bone marrow (98,99), and inhibition of myogenic

differentiation (83). Differentiation of mesenchymal cells into pre-chondroblasts is induced by BMPs but the coordinated progression along the chondroblastic and subsequent osteoblastic lineage is regulated by other growth factors that work in an autocrine or paracrine manner (102). BMPs acting through an autocrine mechanism reduce the expression of collagenase-3 and noggin, thereby inhibiting BMP binding and function and, result in increased production of collagenase-3 (103). Although BMPs exert their action on both osteoblasts and chondroblasts, they do not change the fate of the respective progenitors (104). Early exposure of undifferentiated mesenchymal cells to BMPs induces the chondroblastic pathway, whereas later exposure accelerates osteoblastic differentiation (105). BMPs can stimulate osteoblast differentiation independently of cartilage formation (106). This means that in the case of endochondral bone formation, osteoblasts do not form from a transdifferentiation of chondrocytes but rather as a result of a separate induction (1,70,93,107). Endothelial cells invading the cartilage may serve as a homing target of the stem cells that later develop into pre-osteoblasts (98). Numerous reports show an up-regulation of the osteoblastic phenotype by BMPs. Up-regulation of osteocalcin (OC), osteopontin (OP), osteonectin, bone sialoprotein (BSP), alkaline phosphatase (ALP), receptors for parathyroid hormone, collagen I production and the rate of mineralization are all proof of a promotive effect of BMPs on mesenchyme-derived cells (64,82,86,106,108,109). BMPs can act on various cell types and elicit a response that is specific to that stage of cell differentiation (106). There is evidence that BMPs trigger the production of osteopontin in preosteoblasts whereas in osteoblastic cells osteocalcin is upregulated and bone sialoprotein is expressed in differentiated osteoblasts prior to mineralization (110).

BMPs play an important role in the process of bone modeling and remodeling. The morphogenetic activity of bone matrix is apparent only after its demineralization, which occurs with the controlled action of osteoclasts. Insulin-like growth factors (IGF-I, IGF-II), TGF $\beta$ -1, TGF $\beta$ -2, PDGF, basic and acidic fibroblast growth factors, BMPs and other molecules are produced and become incorporated into the forming bone matrix that serves as a reservoir (111). BMPs bind to collagen type IV (112) or type I (113) and under these conditions are inactive. A heparin-binding site has been identified at the N-terminal segments of the BMP-2 that may function to localize the growth factor and restrict its diffusion (13). Acid treatment associated with osteoclastic action liberates BMPs from their collagenous substrate rendering them biologically active (114) and able to affect cell proliferation and differentiation (70).



Guided streaming of specific cell types into the appropriate pathways makes BMPs important regulators of bone formation (113), with a pivotal role in bone remodeling (110). "Basic Multicellular Units" (BMUs) refer to the functional units of bone in which osteoblasts and osteoclasts act in coordination (115). This is called coupling. During remodeling, which is a "self-maintenance" process, existing bone is resorbed and new bone is deposited. Resorption during each remodeling cycle is balanced by an equal amount of bone formation since the amounts of BMPs and other growth factors released from bone are proportional to the extent of resorption (116,117). There is evidence that BMP-2 promotes expression of cyclooxygenase-2 and the osteoclast differentiation factor in osteoblast-like cells, thus regulating osteoclastogenesis (118). Based on the above, the mitogenic, chemotactic and differentiating effect of BMPs may help to mediate coupling of bone formation to resorption during the adaptive response of remodeling. It supports the cellular components and amplifies the molecular signals needed for the coordinated interaction of various cell types.

### **BMPs in fracture healing**

Fracture healing involves complex interactions among many local and systemic regulatory factors as well as cell types that cluster at the fracture site. Fracture repair represents a situation in which cell differentiation is re-initiated in an otherwise mature organism (2). Mesenchymal stem cells congregate at the area and form a gap-spanning, highly cellular "repair blastema" (119,120). The principal phases during ectopic bone induction are the migration and attachment of progenitor mesenchymal cells, proliferation, differentiation into cartilage or bone cell lineages, mineralization and remodeling, and marrow tissue formation.

The first demarcation of osteoprogenitor cells during fracture repair, referred to as the "stacked-cell layer" (120), is derived from mesenchymal stem cells (119). They are brought into the fracture area under the influence of paracrine or autocrine mechanisms (121) and then differentiate into pre-osteoblasts. In the case of bone fracture where there is a substantial gap between the originally continuous bone, a sequence of cellular and molecular events is initiated in response to the trauma, including inflammation, repair and remodeling (122). Injury leads to blood-clot formation that results in lysis of platelets, releasing numerous growth factors. The blood clot then begins to organize and the formation of a provisional callus that bridges the fracture site becomes apparent. Increased vascular permeability allows fluid and plasma proteins to leave the blood vessels. Various

cell types then emerge from the vessels in significant numbers. In acute inflammation, neutrophils (PMNs) are the first leukocytes to provide an effective defense in response to the trauma.

PGE<sub>1</sub> has a strong and dose-dependent promotive effect on the osteogenic activity of rhBMP (123), and glucocorticoids exert similar effects (69) whereas binding of BMP to free heparin at the fracture site could help to localize the growth factor by restricting its diffusion (13). Another component of the inflammatory process is the acidic conditions that develop and, together with the proteolytic fragments of the plasminogen system, help activate latent forms of growth factors such as TGF- $\beta$ 1 and regulate a positive feed-back system that amplifies activation of TGF- $\beta$ 1 from platelets, thus stimulating cartilage and bone formation (113). BMPs can also induce chemotaxis of monocytes and stimulate their expression of TGF- $\beta$ 1 mRNA (62). Finally, blood-clot formation following tissue injury results in the lysis of platelets that release numerous growth factors that are involved in wound healing and contribute to bone repair (121).

Depending on the mechanical stability of the fracture, mesenchymal stem cells can differentiate into chondroblasts or osteoblasts. If the fracture is mechanically unstable, cartilage will form and the bone fragments will be mechanically joined. If the fracture is mechanically stable, the chondrocytes within the blastema become hypertrophic, and along with their extracellular matrix, become eroded and replaced by osteoblasts and osteoid deposits (119,120). This process is similar to the one observed in embryonic long bone development. If the original break is mechanically stable, the repair blastema can be spanned by vasculature and the mesenchymal cells differentiate directly into secretory osteoblasts (119, 120). Mesenchymal stem cells may originate from the periosteum, the marrow space or are brought to the repair site via the circulatory system. Although the exact origin is not determined, the important fact is that mesenchymal cells will be attracted to the fracture site and play an important role in the repair process (124).

The inflammatory response acts as a multipotential modulator and initiator of the repair process, using mechanisms that will be described later, and as such must be considered a necessary event for the healing of bone wounds. TGF- $\beta$ 1 is an important and multifunctional autocrine regulator of bone formation (125). It has been demonstrated that TGF- $\beta$ 1 downregulates alkaline phosphatase, osteocalcin, osteopontin, collagen I and BMP-2 mRNA expression. This provides evidence that TGF- $\beta$ 1 acts as a powerful bone growth stimulant at the level of pre-osteoblasts (126), which is needed for the

coordinated progression of cell types along their differentiation pathways (127). TGF- $\beta$ 1 stimulates DNA synthesis and replication of osteoprogenitor cells and is chemotactic for mesenchymal cells and osteoblast-like cells for recruitment of osteogenic cells to sites of bone formation and remodeling (113). EGF and FGFs are other molecules with demonstrated involvement in the complex molecular cascades involved in cellular change (128,129).

Implantation of rhBMP-2 resulted in early cartilage formation that was later replaced by bone tissue with bone marrow elements. Invasion of blood vessels into the cartilaginous anlagen lead to bone formation and bone marrow development (98). When larger doses of rhBMP-2 were used, bone formation was observed concurrently with cartilage formation, suggesting bone induction through both endochondral and intramembranous pathways. BMPs are believed to act through chemotactic, mitogenic or differentiating mechanisms. It is important to understand that BMPs are not the only determinants of cell fates along the above-mentioned lineages. Specific nutrients, growth factors and cytokines at specific concentrations and in a specific sequence of exposures are fundamental for the bone formation process.

During fracture healing, BMP-2/4 affect precursor cells to become chondroblasts and express proteins needed for production of woven bone (121). When lamellar bone replaces woven bone, BMP expression is significantly reduced (130). rhBMP-2 can induce bony trabeculae and bone marrow (99) with concomitant shortening of the time required for osteogenesis and increased amount of bone formation (85). BMP-4 is also expressed in less differentiated cells at fracture healing during distraction osteogenesis (131). rhBMP-2 does not increase the mitotic activity of osteoblasts (132) and does not affect DNA synthesis, but rather initiates sequences of gene expression in these cells (133). rhBMP-2 up-regulates the expression of BMP3/4 mRNA (109) with a mechanism that probably augments the transcriptional rate of the gene rather than stabilizing the mRNA (33). Evidence of BMP-4 activating the transcriptional factors *Msx-1/2* and *Egr-1* in epithelial-mesenchymal interactions during tooth development make this mechanism a valid working hypothesis (8). BMPs 1/2/4/6 are expressed by osteoblasts before they form mineralized bone nodules and during expression of ALP, OC, OP (134), thereby becoming guiding factors in osteoprogenitor cells (106). Unlike the BMPs, the TGF $\beta$ s do not induce ectopic bone formation and inhibit chondrogenesis (63,110) but promote bone healing and fracture repair (125,135). However, acting at the level of cell adhesion molecules (36), they may stimulate mesenchymal cell attraction and proliferation (136). Being

part of a BMP-TGF $\beta$  heterodimer can amplify the BMP effect (137) especially at the early stages of the bone repair process (61, 138). BMP-2 up-regulates the phenotypic expression of osteoblasts (82,83,133,139) and may indeed antagonize the repressing action of TGF- $\beta$  by cross-reacting with TGF- $\beta$  receptors (132). Additional studies have demonstrated a promotive effect of rhFGF (140) or prostaglandin E1 (PGE1) (123) on the action of BMPs, reducing the amount of growth factor needed to elicit a specific biologic response (141).

## Carriers

The majority of studies investigating the role and action of exogenous BMPs use a matrix to deliver the growth factor to the implantation site. Although the matrix may not contribute any additional factors necessary for bone induction (107), it is a fundamental and very important component of the growth process. Collagenous or synthetic matrices have been used as delivery vehicles and their physicochemical properties, together with the microenvironment they create, play a role in the inductive outcome. Carriers can be solid xenogenic (HA) (89,142), solid alloplastic (polyethylene polymers) materials (143,144), or gels of autogenous (88,145), allogenic (146,147), or alloplastic origin (148), and combinations of the above (149).

One of the carrier functions is to maintain the factor at the site of implantation and thus enhance its local concentration. However, BMPs also help to stabilize the carrier by accelerating bone growth in its mass (150) due to the stabilization brought about by the BMPs absorption to the surface of the carrier matrix particles. As a result, 0.15 $\mu$ g of rhBMP-2 with matrix induced bone formation subcutaneously in rats, while a minimum of 75  $\mu$ g of rhBMP-2 was required in the absence of matrix (11). The isoelectric point and the structural features of the protein are important determinants of the implant-retained dose but the pharmacokinetics of the growth factor are not affected by carrier properties (151). Collagen matrix retains ~65% of the BMPs during initial impregnation and releases it in two phases: an initial phase within hours of implantation and a second phase that depends on the nature of the carrier and its geometrical characteristics (152).

It is believed that BMPs do not bind to the carrier (152), but rather become physically entrapped in its structure which makes certain designs more favorable for bone induction over some others (153). In the case of collagen sponge carriers, the mass, collagen cross-linking and sterilization methods affect BMP precipitation and subsequent resistance of sponge degradation by collagenase (154). Properties of the best carrier may vary depending

on the specific implantation site and the intended therapeutic outcome. Considerations include biodegradability, structural integrity, absence of immunogenicity, absorption and rate of release of BMP (155). The latter characteristic of the carrier serves its second function, which is controlled release of the BMP. This allows for a more constant and prolonged application. This renders BMPs more efficient and helps to create the chemotactic gradient, necessary for the cells to respond (11). BMP-2 is retained in a hydrogel carrier for more than 30 days whereas direct injection results in its complete elimination within 3 days (156). Collagen carrier also resulted in increased bone density of the regenerate when compared to polymeric matrix (157), emphasizing the importance of the structural properties of the carrier.

Recently, a novel approach has been suggested. This involves implanting matrices that actively concentrate native BMPs at the implantation site instead of passively storing and delivering rhBMPs which are a thousand times less potent than the native BMP complex (158). The matrix also serves as an environment in which bone can form and therefore helps to define the region in which new bone can be formed (159). Delivery vehicles with adequate structural consistency can function as primary scaffolds on which cells can attach and ECM, with subsequent mineralization, can be deposited (152,160). If the delivery matrix can act as a scaffold, then the cartilaginous intermediate may not be necessary. Many investigators agree that it has not been proved definitively that the chondrogenic process is essential for bone formation by BMP (89,161). The type of matrix used may also influence and determine the mechanism of bone formation that is appropriate for the implantation site (146). The material of the matrix and its geometrical parameters (pore size, and %volume) are factors that directly (size of cells able to attach) or indirectly (effect on blood or oxygen supply) determine the micro-environment and influence the mechanism of bone formation (endochondral or intramembranous) (59,89,90). BMPs combined with porous particles of hydroxyapatite or fibrous collagen membrane lead to intramembranous ossification (89,142,161), whereas fibrous glass membrane or insoluble bone matrix support indirect bone formation *via* a cartilaginous intermediate (89,90,142).

In examining the action of BMPs, it is also important to consider dose-related effects. It is evident that various doses elicit different responses on specific cell types at different time intervals (162). The dose of the growth factor determines its chemotactic, proliferative or mitogenic signal and should therefore be well regulated. Increased BMP concentrations result in faster bone growth (11), with cartilage being more rapidly replaced by mineralized

osteoid (163). rhBMPs in the form of monomers, homodimers or heterodimers need to be evaluated and standardized because they exhibit different biological potencies (11,121).

The carrier may also act synergistically by serving as a reservoir of the inducible cell population. Bone marrow can be combined with BMPs (164) and, when providing its cellular component, can result in bone formation of superior performance. Recently, investigators attempted the direct (*in vivo*) or indirect (using viral vectors) delivery of BMP genomic sequences to the implantation site (165,166), demonstrating active BMP expression for 2-6 weeks and bone formation with trabeculae and bone marrow. Cost of manufacturing and handling, in addition to ease of clinical application, are equally important factors to consider when deciding on a specific type of delivery vehicle.

## Clinical applications

BMPs are of tremendous interest as therapeutic agents for healing bone fractures, preventing osteoporosis, treating periodontal bone defects and enhancing bone response around alloplastic materials implanted in bone (3). rhBMP-2 delivered with an absorbable collagen sponge (ACS) has been used for the augmentation of the maxillary sinus floor in humans (167). An rhBMP-2 dose ranging from 1.77 to 3.4mg per patient generated an average of 8.51mm of vertical bone height in four months providing a promising alternative to traditional grafting procedures (167). Similar results were also achieved in sub-antral augmentation of non-human primates with 6 mm of vertical bone gain and increased density that allowed placement of titanium implants (168). BMP-2 regenerated bone in irradiated tissues also provides the clinical potential to treat patients who have undergone radiation therapy and need bone reconstruction (169).

Periodontal regeneration was achieved when rhBMP-2 was applied to the defect site with a collagen membrane or a collagen gel. However, better results were obtained using the slower dissolving collagen membrane that allowed delivery of the growth factor for a prolonged period of time (170). The clinical outcome was a decreased depth of the defect site brought about by stimulating vertical bone growth and regenerating the periodontal attachment, provided that adequate space is maintained (171-175). The type of carrier, the time of treatment and the use of a barrier membrane are critical factors influencing the therapeutic outcome in cases of bone regeneration around dental implants (157) and have been shown to produce accelerated healing time as well as improved bone-implant contact levels (175-179). Moreover, alveolar ridge

preservation or localized augmentation have been documented in humans (180).

Animal studies also suggest that rhBMP-2/ACS may be an effective treatment for the restoration of segmental bone defects (181,182) and could lead to increased callus volume (183), strength and stiffness (184). A bioerodible polymeric carrier was used to deliver rhBMP-2 in a large segmental defect that was stabilized with stainless steel plates (185). Stabilization was necessary because of the large size of the animals (sheep) but it could have also helped to provide a stable environment for bone bridging since the carrier was reported to fragment easily. In a similar study in rabbits, porous poly-lactic acid carrier combined with rhBMP-2 was found to restore cortical bone with marrow elements in a twenty-millimeter long segmental defect (186). Skull defects were also filled with regenerated bone when BMPs were delivered in combination with hydroxyapatite (2,187), a biodegradable gelatin hydrogel or an aqueous solution (188). Spinal fusion was significantly enhanced when rhBMP-2 was administered with a hydroxyapatite graft or a collagen gel, and demineralized bone matrix revealed improved biomechanical properties and enhanced radiographic and histologic appearance (189).

Although purification and characterization of rhBMP-2 has been described in the Chinese hamster ovary (CHO) cell line (64) rendering BMPs available in large quantities, the fact that their inductive activity is ten times less than that of purified BMPs may present a limitation for their clinical application (190). Combinations of BMPs with other growth factors or biologic molecules forming heterodimers with twenty times higher potency in some cases (191) than homodimeric forms, hold a promising future in the field of bioengineering.

The parameter of host age further affects the biologic potential of many growth factors (161). The bone inductive ability of BMP-2 is diminished in older organisms and higher doses are required to induce the bone formation effect (192). Reduced migration of mesenchymal cells, lower levels of local anabolic agents, age associated reduction of receptor levels and compromised vascularization are some of the aspects to take into consideration (193-195). In the future, delivery of biological agents that control the regulators of BMPs may be of clinical significance in cases where BMP action needs to be halted to prevent pathological or hazardous ossification, such as after total hip or temporomandibular arthroplasties (44). Production of natural autogenous bone in moulds may allow a more efficient reconstruction of defects and deformities.

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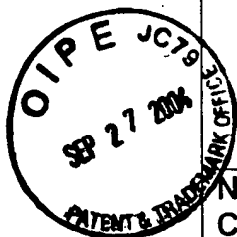
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## Notice to Comply

Application No.

10/005228

Examiner

M. Marvich

Applicant(s)

LYONS et al.

Art Unit

1636

### NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Applicant must file the items indicated below within the time period set the Office action to which the Notice is attached to avoid abandonment under 35 U.S.C. § 133 (extensions of time may be obtained under the provisions of 37 CFR 1.136(a)).

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

- ☒ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).
- ☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).
- ☐ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).
- ☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."
- ☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).
- ☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable form of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).
- ☒ 7. Other: There are sequences on page 20, 21, 22, 34 and 35 as well as a blank following SEQ ID on page 8.

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